Remarks

Status of the Claims and Support for the Amendments to the Claims

By the foregoing amendments, claim 1 is sought to be amended. Support for the amendment to claim 1 can be found throughout the specification. Therefore, this amendment introduces no new matter. Upon entry of the foregoing amendment, claims 1-4, 7, 8, 12, 69, 73 and 75-76 are pending in the application, with claims 1 and 73 being the independent claims.

Summary of the Office Action

In the Office Action dated December 6, 2006, the Examiner has made four rejections of the claims. Based on the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

The Rejection Under 35 U.S.C. § 103(a) Over Yu in view of Marks, Wright and Morishige

In the Office Action at pages 2-4, section 5, the Examiner has rejected claims 1, 3, 7, 8, 12, 73, 75 and 76 under 35 U.S.C. § 103(a), as allegedly being unpatentable over Yu *et al.*, *Oncogene 11*:1383-1388 (1995) (hereinafter "Yu") in view of Marks *et al.*, U.S. Published Patent Application No. 2001/0008759 (hereinafter "Marks"); Wright and Huang, *Biochim. Biophys. Acta. 1103*:172-178 (1992) (hereinafter "Wright"); and Morishige *et al.*, *Biochim. Biophys. Acta 1151*:59-68 (1993) (hereinafter "Morishige"). Applicants respectfully traverse this rejection.

The Examiner contends that Yu discloses cationic liposome-mediated E1A gene transfer, and the use of anti-p185 antibodies to construct immunoliposomes. The Examiner also contends that Yu discloses using a DNA:liposome ratio of 1:13, which allegedly falls within the range recited in present claim 1. The Examiner states that Yu does not disclose the use of an antibody fragment, including scFv, nor the ratio of incorporation of antibodies into the liposomes. The Examiner also states that Yu does not disclose direct conjugation between an antibody fragment and a liposome via a sulfur atom that was part of a sulfhydryl group at a carboxy terminus of the scFv. The Examiner relies on the disclosures of Marks, Wright and Morishige to cure these deficiencies.

The Examiner asserts that Marks discloses the use of scFv antibodies with a free cysteine reside at the C-terminus of the scFv for the preparation of targeted immunoliposomes. With regard to Wright, the Examiner contends that this reference discloses that an antibody can be attached to MPB-PE that has been used to stabilize the bilayer phase of DOPE liposomes in order to generate target sensitive immunoliposomes. Finally, the Examiner asserts that Morishige discloses conjugating Fab' fragments with liposomes containing MPB-PE at a ratio of 1 mg Fab' per 6 μmol of PC. The Examiner asserts that this allegedly falls within the range recited in present claim 1. Applicants respectfully disagree with the Examiner's assertions and contentions.

The Examiner asserts that it would have been obvious to combine these references in order to make a targeted cationic liposome containing a DNA therapeutic agent capable of targeting the immunoliposome to a Her2/neu expressing tumor. The Examiner contends that: 1) Yu discloses cationic liposome-mediated gene transfer and the attachment of an anti-Her2neu antibody to the liposomes; 2) Marks discloses anti-Her2/neu scFV antibody

fragments, and that such fragments are preferred; 3) Wright discloses the attachment of antibodies to MPB-PE; and 4) Morishige discloses a coupling ratio of antibody to liposome that allegedly falls within the ratios recited in the presently claimed invention. The Examiner therefore concludes that it would have been obvious for one of ordinary skill in the art at the time of filing to combine these references in the required manner. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As set forth in *Graham v. John Deere Co. of Kansas City*, "[u]nder § 103, the scope and content of the prior art to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined." 383 U.S. 1, 17 (1966). Applicants respectfully submit that the differences between the presently claimed invention and the references cited by the Examiner are so great that it would not have been obvious to combine the various citations, as required by the Examiner, in order to render the presently claimed invention obvious.

Applicants respectfully submit that Yu does not disclose an antibody-fragment-targeted cationic immunoliposome complex, wherein the antibody fragment is directly conjugated to the cationic liposome, or the use of a sulfur atom which was part of a sulfhydryl group for such conjugation. In fact, Yu does not disclose an antibody fragment-targeted immunoliposome, or any method for preparing such a liposome (including mixing an antibody prior to or after mixing with DNA). Yu simply mentions in passing that liposomes could be targeted to the HER-2/neu-encoded p185 receptor. Yu provides no disclosure of the methods, ratios of lipid to protein, conditions, or other requirements for creating such immunoliposomes. In fact, Yu admits that such targeted liposomes could only

be prepared once a ligand for the HER-2/neu-encoded p185 receptor becomes available (see Yu at page 1387, column 1, last line of the first full paragraph). However, Yu clearly does not disclose or enable any method for preparing such immunoliposomes, much less the immunoliposomes of the present invention. Applicants respectfully submit that these deficiencies are not cured by the disclosures of Marks, Wright or Morishige, alone, or in combination.

The Examiner contends that Marks discloses coupling of anti-ErbB2 svFvs to liposomes to prepare immunoliposomes containing chemotherapeutics. Applicants submit, that while Marks mentions that scFv molecules were coupled to liposomes, there is no indication in Marks as to whether the scFvs were directly conjugated to the liposome, or attached via a poly(ethylene glycol) or other linker molecule. Furthermore, Marks does not disclose whether the liposomes that are being utilized are cationic or neutral liposomes. However, the reference cited at page 18, paragraph 206 of Marks, Kirpotin *et al.*, *Biochemistry 36*:66-75 (1997) (attached herewith as Exhibit A), indicates that the lipids used in the disclosed experiments were palmitoyloleoylphosphatidylcholine (POPC), a neutral lipid.

As discussed in greater detail below, Applicants respectfully submit that cationic liposomes, such as those utilized in the presently claimed invention (and in Yu) have very different properties and characteristics that do not directly correlate with those of neutral liposomes. Marks does not provide any specific disclosure with regard to the methods by which the scFvs are coupled to the vesicles, and in addition, does not disclose the use of cationic liposomes. In addition, the disclosure of Marks is limited to chemical-containing liposomes (doxorubicin), and does not make any mention of the addition of nucleic acids to

the liposomes. One of ordinary skill in the art practicing the methods of Yu would not have combined this disclosure with that of Marks, as the differences between the properties and characteristics of neutral liposomes (the subject matter of Marks) and cationic liposomes (the subject matter of Yu and the presently claimed invention) are so great, that combining these references would not have yielded predictable results, and in fact, a person of ordinary skill in the art at the time of filing of the present invention would have been directed away from making such a combination. (*See, KSR Int'l. Co. v. Teleflex Inc.*, 550 U.S. ___, WL 1237837 (April 30, 2007) ("a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions.")).

Applicants respectfully submit that combining the disclosure of Yu with the disclosure of Marks, or either of the disclosures of Wright or Morishige, in order to produce the presently claimed invention, would not have been a predictable use of these references. As noted above, Yu is directed toward the use of cationic liposomes to which nucleic acids are added in order to produce gene-transfer agents. The positive charge on the surface of the liposomes allows for an electrostatic interaction between the liposomes and the negatively charged DNA. Based on the statement in Yu, "one of our next efforts should be designing liposomes that can target the E1A gene to tumors," the Examiner contends that one of ordinary skill in the art would have sought to utilize the methods disclosed in Marks, Wright and Morishige to target these liposomes, and hence, the presently claimed invention is rendered obvious. Applicants respectfully submit, as detailed below, that several years after the disclosure of Yu (and approximately one year prior to Applicants' filing of 35 U.S.C. § 120 priority application, PCT/US00/0432), persons of ordinary skill in the art were still struggling with how to target cationic liposomes. Furthermore, those who were

working to solve the problem clearly would not have looked to disclosures utilizing noncationic liposomes.

Attached herewith is a copy of Li and Huang, "Functional Pleomorphism of Liposomal Gene Delivery Vectors, Lipoplex and Lipopolyplex," Liposomes. Rational Design, Ed. A.S. Janoff, Marcel Dekker, Inc., New York, 1999, Chapter 4, pp. 89-124 (attached herewith as Exhibit B; hereinafter "Li and Huang"). Applicants note that one of the authors of Li and Huang, Dr. Leaf Huang, is also an author of Yu.

At page 119, Section D, second full paragraph, Li and Huang describe some of the problems that still faced cationic liposome delivery at that time. "Another problem with these formulations is the difficulty of tissue-specific gene delivery. . . . Despite this limitation, there are many potential applications for systemic use of cationic liposomes/DNA complexes. In these applications, delivery of gene to target cells via cationic lipid vectors is achieved mainly through their *intrinsic properties of non-specific interaction* with pulmonary endothelial cells" (emphasis added). Thus, several years after the publication of Yu, scientists in the field of cationic liposome-based gene delivery (specifically one of the *same* scientists who identified "the next effort" in Yu) were still searching for methods to specifically target these carriers, and were primarily relying on the intrinsic, non-specific interactions between the liposomes and the target tissue. Applicants respectfully submit that this clearly demonstrates that the long felt need discussed in Yu, was still an unsolved need several years later, despite the presence of the references cited by the Examiner that allegedly disclosed targeting *noncationic* liposomes (i.e., Wright and Morishige). As noted in *Graham*, "[s]econdary considerations such as commercial success, *long felt by unsolved*

need, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." 35 U.S. at 17-18 (emphasis added).

Li and Huang also state the following:

> Development of long-circulating lipid vectors, together with the use of a targeting ligand *might* provide a solution to the problem. It should be noted, however, that DNA is a large molecule with large hydrodynamic diameter as compared with chemotherapeutic drugs.

Li and Huang at page 120, lines 1-7 (emphasis added). Applicants respectfully submit that this not only demonstrates that targeting of DNA-liposome vectors was clearly unsolved, but that furthermore, one of ordinary skill in the art would not have predicted that antibody or antibody fragments could be directly conjugated to cationic liposomes comprising DNA attached to their surface (i.e., the "DNA:liposome" cationic liposomes of Yu). Applicants respectfully submit that in fact, Li and Huang actually teach away from directly conjugating antibody fragments (or antibodies) to liposomes comprising DNA on their surface. As stated in Li and Huang, "DNA is a large molecule with large hydrodynamic diameter as compared with chemotherapeutic drugs." One of ordinary skill in the art would not have predicted that additional molecules, such as antibodies or antibody fragments, could then *further* be added to these liposomes. The presence of large hydrodynamic diameter molecules on the surface of a cationic liposome, such as that disclosed in Yu, would have lead one of ordinary skill in the art *away* from disclosures where antibodies or antibody fragments are directly conjugated to liposomes, such as noncationic liposomes that *do not* comprise DNA on their surface, but rather encapsulate drugs or other agents (as disclosed in Marks, Wright and Morishige).

Thus, the disclosure of Li and Huang actually shows that the combination of Yu with any of the disclosures of Marks, Wright or Morishige, to produce a targeted cationic

nucleic acid-comprising liposome, would have been contrary to the knowledge of one of ordinary skill in the art. As set forth in M.P.E.P. § 2145(X)(D)(3), proceeding contrary to accepted wisdom in the art is evidence of nonobviousness. *In re Hedges*, 783 F.2d 1038 (Fed. Cir. 1986). Furthermore, one of ordinary skill in the art would not have predicted that cationic liposomes could be targeted by directly conjugating an antibody fragment to the liposome, as recited in the presently claimed invention. Hence, the presently claimed invention represents more than the predictable use of the references cited by the Examiner. *See KSR,* U.S. 550 at 13.

As noted above, the Examiner contends that Wright discloses that thiolated antibodies can be attached to liposomes containing MPB-PE, and that such methods facilitate proper orientation of the antibody and avoids the use of detergent that is employed with acylated antibody. Assuming *arguendo* that Wright discloses the attachment of antibodies to MPB-PE lipids in a liposome, Wright makes no mention of the attachment of scFvs to such groups. Applicants respectfully submit that the ordinarily skilled artisan would not have predicted that scFvs could be used in place of full length antibodies due to their differences in size, structure and properties of the two classes of molecules.

Furthermore, as discussed above, one of ordinary skill in the art would not have predicted that the methods of targeting noncationic liposomes, as disclosed in Marks, Wright, or Morishige could have been utilized with the DNA-comprising cationic lipids of Yu. The disclosure of Wright is limited to neutral liposomes (dioleoylphosphatidylethanolamine (DOPE)), focusing on the use of MPB-PE as a vesicle stabilizer. There is no mention that the liposomes in Wright could be used in combination with nucleic acids, as recited in the presently claimed invention, and as utilized in Yu.

In addition, Dr. Leaf Huang, co-author of Li and Huang, is also co-author of Wright. Applicants respectfully submit, if Dr. Huang, who in 1992 (publication date of Wright) allegedly disclosed that antibodies could be attached to the surface of liposomes using MPB-PE molecules, then disclosed in 1995 (publication date of Yu) that "our next efforts should be designing liposomes that can target," and in 1999 (publication date of Li and Huang), was *still searching* for a solution to the problem of "tissue-specific gene delivery," how could such a combination, as required by the Examiner, be *obvious* to one of ordinary skill in the art. This clearly does not, and can not, represent a *predictable* use of elements in the art. *See KSR*, 550 at 13.

Finally, the Examiner contends that one of ordinary skill in the art would have utilized the ratios of Fab' to liposome disclosed in Morishige when preparing liposomes comprising scFvs. Applicants respectfully submit that Morishige is limited to the use of Fab' fragments, and makes no mention of the use of scFvs. Fab' fragments are much larger molecules that scFvs, and hence, there is no reason to believe that the same ratios could be utilized in the preparation of scFv-comprising immunoliposomes. Furthermore, the Examiner again has attempted to combine the disclosure of Yu with a reference, Morishige, that discloses only the use of neutral liposomes (phosphatidylcholine/cholesterol). As discussed above, a person of ordinary skill in the art would not have predicted that the ratios disclosed in Morishige for use with noncationic lipids could have been utilized with the cationic liposomes of Yu. In fact, as noted in Li and Huang, a person of ordinary skill in the art would not have considered the disclosure of Morishige instructive when preparing DNA-bound cationic liposomes, as Morishige does not utilize such liposomes, but rather, discloses only adding Fab' fragments to neutral liposomes with no concern of the effects of the large

hydrodynamic radii of surface DNA molecules. Thus, it would not have been predictable to combine these references in order to render the presently claimed invention obvious.

In view of the foregoing remarks, Applicants respectfully submit that the Examiner has not provided sufficient evidence that the presently claimed invention has been rendered obvious by the disclosures of Yu, Marks, Wright or Morishige, alone or in combination. Applicants submit that the differences between the presently claimed invention and the cited references are so great that one of ordinary skill in the art would not have predicted that the required elements could be combined to generate the presently claimed invention. *See KSR*, 550 U.S. at 13. Furthermore Applicants have submitted evidence of a long felt but unmet need in the art (*see Graham*, 383 at 17), as well as disclosure that the required combination would have proceeded contrary to accepted wisdom in the art (*see Hedges*, 783 F.2d 1038). Hence, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 3, 7, 8, 12, 73, 75 and 76 under 35 U.S.C. § 103(a).

The Rejection Under 35 U.S.C. § 103(a) Over Yu in view of Marks, Park and Papahadjopoulos

In the Office Action at pages 4-7, section 6, the Examiner has rejected claims 1, 3, 7, 8, 12, 73, 75 and 76 under 35 U.S.C. § 103(a), as allegedly being unpatentable over Yu in view of Marks; Park *et al.*, Advances in Pharmacology 40:399-435 (1997) (hereinafter "Park"); and Papahadjopoulos *et al.*, Published U.S. Patent Application No. 2004-0209366 (hereinafter "Papahadjopoulos"). Applicants respectfully traverse this rejection.

As noted above, the Examiner contends that Yu discloses cationic liposome-mediated E1A gene transfer, and the use of anti-p185 antibodies to construct

immunoliposomes. The Examiner also contends that Yu discloses using a DNA:liposome ratio of 1:13, which allegedly falls within the range recited in present claim1. The Examiner states that Yu does not disclose the use of an antibody fragment, including scFv, nor the ratio of incorporation of antibodies into the liposomes. The Examiner also states that Yu does not disclose direct conjugation between an antibody fragment and a liposome via a sulfur atom that was part of a sulfhydryl group at a carboxy terminus of the scFv. The Examiner relies on the disclosures of Marks, Park and Papahadjopoulos to cure these deficiencies.

As discussed above, the Examiner asserts that Marks discloses the use of scFv antibodies with a free cysteine reside at the C-terminus of the scFV for the preparation of targeted immunoliposomes. With regard to Park, the Examiner contends that this reference discloses that an Fab' can be directly conjugated to maleimido-phosphatidylethanolamine, resulting in Fab' directly linked to the liposome surface, and further that Park discloses linking scFv fragments to liposomes. Finally, the Examiner asserts that Papahadjopoulos discloses the use of antibody fragments, including scFvs, as targeting molecules for cationic liposomes. The Examiner specifically asserts that Papahadjopoulos discloses that scFvs can be bound to liposomes through cysteine residues. The Examiner also contends that Papahadjopoulos discloses ratios of DNA to lipid, and antibody to lipid, that fall within the scope of the presently claimed invention. Applicants respectfully disagree with the Examiner's assertions and contentions.

The Examiner then concludes that it would have been obvious to one of ordinary skill in the art at the time the invention was made to prepare a liposome composition as disclosed in Yu, comprising the anti-ErbB2 scFv disclosed in Marks, using the direct

coupling method disclosed in Park, at the ratios disclosed in Papahadjopoulos. Applicants respectfully disagree with the Examiner's conclusions.

As discussed above, Applicants respectfully submit that Yu does not disclose any methods for the targeting of nucleic acid/cationic liposomes. Furthermore, Marks also does not disclose any specific methods for attaching an scFv directly to a liposome, and does not indicate that such methods could be utilized with cationic liposomes, as required by the presently claimed invention, and as utilized in Yu.

With regard to Park, Applicants respectfully submit that the conjugation of Fab' fragments directly to the surface of a liposome does not render obvious the direct conjugation of scFvs. As discussed above, the differences between the sizes and characteristics of Fab' fragments and scFvs are such that one of ordinary skill in the art would not consider it predictable to substitute one molecule for the other. In addition, the linkage of scFvs to antibodies discussed in Park is limited to the use of lipid-tagged scFvs, not direct conjugation between an antibody fragment and a cationic liposome where the conjugation occurs via a sulfur atom which was part of a sulfhydryl group at a carboxy terminus on the antibody fragment prior to the conjugation. The reference cited on page 405 of Park, last six lines of the second full paragraph, Laukkanen *et al.*, *Biochemistry 33*:11664-11670 (1994) (attached herewith as Exhibit C) indicates that the single-chain antibodies were expressed in *E. coli* as lipid-tagged molecules. (*See* Laukkanen *et al.*, abstract). Hence, Applicants respectfully submit that Park does not provide a disclosure sufficient to render obvious the presently claimed invention.

Furthermore, as noted above, Yu and the presently claimed invention require the use of cationic liposomes. The disclosures of Marks and Park are limited to noncationic

liposomes. (*See* Park at page 409, lines 8-11, "(phosphatidylcholine plus cholesterol:PC/Chol); and Laukkanen *et al.*, at page 11665, right hand column, lines 3-6, "pure egg yolk phospholipid/cholesterol mixture (10 mg, PC/PE/Cho, 10:1:5 molar ratio)." Phosphatidylcholine and pure egg yolk phospholipid are both noncationic liposomes.) In addition, neither Marks nor Park disclose the targeting of nucleic-acid comprising cationic liposomes. Thus, Applicants respectfully submit that the differences between the presently claimed invention, directed toward the direct conjugation of single chain antibody fragments to cationic liposomes that also comprise nucleic acid, and the references cited by the Examiner are significant. As discussed in detail above with regard to Li and Huang, it would not have been predictable that the methods disclosed in Marks or Park , limited to noncationic liposomes, could be utilized in combination with the disclosure of Yu, requiring cationic liposomes, to generate the presently claimed invention.

With regard to Papahadjopoulos, as stated in Applicants' Reply to Office Action filed on March 30, 2006, the disclosure of which is incorporated herein by reference in its entirety, Applicants respectfully submit that this reference does not disclose a nucleic acid-cationic immunoliposome complex in which an scFv antibody fragment is directly conjugated to a cationic liposome via a sulfur atom which was part of a sulfhydryl group at a carboxy terminus on the antibody fragment, as recited in present independent claims 1 and 73. All of the targeting examples in Papahadjopoulos require the use of a polymer (PEG) linker (Maleimido-propionylantido-PEG-diastearoylphosphatidylethanolamine (Mal-PEG-DSPE)) to link the antibody fragment to the liposome via a hydrophobic interaction. Papahadjopoulos thus does *not* disclose direct conjugation of the antibody to a cationic liposome, as required in the presently claimed invention.

In addition, Applicants respectfully submit that Papahadjopoulos does not disclose the 1:5 to 1:40 w/w ratio of protein:lipid recited in present claim 1. The Examiner refers to Example 7 of Papahadjopoulos, stating that the liposome utilized in this Example is made of the same constituents as the liposome in Example 6, and contends that the ratio of antibody fragment to lipid of 15.6 μg:1 μmol (or 1 μg:64 nmol), falls within the scope of claim 1. Applicants respectfully disagree with the Examiner's contentions.

As set forth in Applicants' previously filed reply:

> The wt:wt (e.g, μg) ratios of scFv:liposome in present claim 1 of
> 1 μg:5μg to 1 μg:40 μg, correspond to molar ratios of
> 0.036 nmol protein:7 nmol lipid to 0.036 nmol protein:56 nmol lipid.
> Or, utilizing wt:mol values, the ratios of present claim 1 correspond to
> 1 μg protein:7 nmol lipid to 1 μg protein:56 nmol lipid.

This conversion clearly demonstrates that the ratio of 1 μg of protein:64 nmol lipid recited in Example 7 of Papahadjopoulos falls outside the scope of present claim 1.

To further confirm that the ratios disclosed in Papahadjopoulos do not fall within the scope of present claim 1, the ratio of 15.6 mg protein:1 μmol lipid can be converted to a wt:wt ratio as follows.

Assuming that the lipid composition utilized in Example 7 of Papahadjopoulos is the same composition set forth in Example 6, this composition comprises:

1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) (MW:760.09g/mol);

Cholesterol (MW:386.66 g/mol); and

Methoxypolyoxyethyleneglycol-derivatized disearoyl phosphatidylethanolamine (DSPE-PEG) (MW:1900 g/mol (PEG) + 748.08 g/mol (DSPE) ≅ 2648.08 g/mol).

The lipids are mixed at a molar ratio of 30:20:3 (POPC:Cholesterol:DSPE-PEG).

Utilizing the 1 μmol of lipid from Example 7, the molar amounts of each component would therefore be:

> 0.569 μmol POPC:0.379 μmol cholesterol:0.0569 μmol DSPE-PEG).
> Converting these molar values to weights (based upon the molecular weights above) 0.433 mg POPC:0.147 mg cholesterol:0.151 mg DSPE-PEG
> The total weight of 1 μmol of liposome is therefore 731 μg of lipid.
> The ratio of 15.6 μg of protein: 1 μmol lipid utilized in Example 7 is then 15.6 μg protein:731 μg of lipid, which reduces to 1 μg protein: 48.7 μg lipid.

This wt:wt ratio of 1:48.7 is clearly outside of the range disclosed in present claim 1 of 1:5 to 1:40.

Hence, Papahadjopoulos does not disclose the protein:lipid ratios recited in present claim 1, and hence, does not render obvious present claim 1. In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1, 3, 7, 8, 12, 73, 75 and 76 under 35 U.S.C. § 103(a).

The Rejection Under 35 U.S.C. § 103(a) Over Yu in View of Marks, Wright and Morishige, and Further in View of Xu and Scherman

In the Office Action at pages 7-8, section 7, the Examiner has rejected claims 2, 4 and 69 under 35 U.S.C. § 103(a), as allegedly being unpatentable over Yu in view of Marks; Wright; and Morishige; and further in view of Xu *et al.*, *Human Gene Therapy 8*:467-475 (1997) (hereinafter "Xu"); and Scherman *et al.*, U.S. Patent No. 6,200,956. Applicants respectfully traverse this rejection.

As discussed above, the Examiner asserts that Yu, Marks, Wright and Morishige disclose the presently claimed invention, with the exception of an antibody fragment that is

capable of binding to a transferrin receptor and a nucleic acid that encodes a wild type p53. The Examiner relies on the disclosures of Xu and Scherman to cure these deficiencies.

With regard to Xu, the Examiner asserts that Xu discloses the use of transferrin-cationic liposomes for delivery of wild type p53 to various tumors. The Examiner also contends that Scherman discloses immunoliposomes comprising transferrin and transferrin antibodies/fragments as targeting molecules for cells such as tumor cells. The Examiner concludes that it would have been obvious for one of ordinary skill in the art to combine these various disclosures to have made the immunoliposomes of the presently claimed invention for delivery of a p53 gene, using a scFv antibody fragment with a specificity for transferrin coupled directly to the liposome, based upon the various disclosures of Yu, Marks, Wright, Morishige, Xu and Scherman. Applicants respectfully disagree with the Examiner's conclusion and the contentions on which they are based.

With regard to Xu, Applicants note that the reference does not disclose the use of scFv fragments, disclosing instead liposomes complexed with transferrin, as a targeting ligand. Transferrin, and an scFv antibody fragment, such as the anti-transferrin receptor scFv used in examples of the present application, are very different molecules, with different sizes and very different functions. One of ordinary skill in the art would have predicted that one could be substituted for the other.

Finally, Scherman does not disclose conjugation of scFv fragments to liposomes. The reference does not disclose direct conjugation, including conjugation via a sulfhydryl group, nor what ratios of protein and lipid would be required to prepare the cationic immunoliposomes of the present invention.

Applicants respectfully submit that the ordinarily skilled artisan would not have predicted that any of these references could have been utilized in combination with Yu, let alone to produce the presently claimed invention. As discussed throughout, one of ordinary skill in the art would not have predicted that methods for targeting noncationic, non-nucleic acid-comprising liposomes could be used to target the liposomes disclosed in Yu, and in fact, such a combination would have proceeded contrary to the excepted wisdom in the art at the time. Neither the disclosure of Xu, nor the disclosure of Sherman, are able to cure the deficiencies described above. Hence, Applicants respectfully submit that the presently claimed invention has not been rendered obvious by the combination of these references.

In view of the foregoing remarks, Applicants respectfully submit that claims 2, 4 and 69 are not rendered obvious by the disclosures of Yu, In View of Marks, Wright, Morishige, Xu and Scherman, alone, or in combination. Hence, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

The Rejection Under 35 U.S.C. § 103(a) Over Yu in View of Marks, Park and Papahadjopoulos, and Further In View of Xu and Scherman

In the Office Action at pages 9-10, section 8, the Examiner has rejected claims 2, 4 and 69 under 35 U.S.C. § 103(a), as allegedly being unpatentable over Yu; in view of Marks; Park and Papahadjopoulos; and further in view of Xu; and Scherman. Applicants respectfully traverse this rejection.

As discussed above, the Examiner asserts that Yu, Marks, Park and Papahadjopoulos disclose the presently claimed invention, with the exception of an antibody fragment that is

capable of binding to a transferrin receptor and a nucleic acid that encodes a wild type p53. The Examiner relies on the disclosures of Xu and Scherman to cure these deficiencies.

With regard to Xu, the Examiner asserts that Xu discloses the use of transferrin-cationic liposomes for delivery of wild type p53 to various tumors. The Examiner also contends that Scherman discloses immunoliposomes comprising transferrin and transferrin antibodies/fragments as targeting molecules for cells such as tumor cells. The Examiner concludes that it would have been obvious for one of ordinary skill in the art to combine these various disclosures to have made the immunoliposomes of the presently claimed invention for delivery of a p53 gene, using a scFv antibody fragment with a specificity for transferrin coupled directly to the liposome, based upon the various disclosures of Yu, Marks, Park, Papahadjopoulos, Xu and Scherman. Applicants respectfully disagree with the Examiner's conclusion and the contentions on which they are based.

With regard to Xu, as discussed above, Applicants note that the reference does not disclose the use of scFv fragments, disclosing instead liposomes complexed with transferrin, as a targeting ligand. Transferrin, and an scFv, such as the transferrin receptor scFv used in examples of the present application, are very different molecules, with different sizes and very different functions. One of ordinary skill in the art would have predicted that one could be substituted for the other.

As noted above, Scherman does not disclose conjugation of scFv fragments to liposomes. The reference does not disclose direct conjugation, including conjugation via a sulfhydryl group, nor what ratios of protein and lipid would be required to prepare the cationic immunoliposomes of the present invention.

Applicants respectfully submit that the ordinarily skilled artisan would not have predicted that any of these references could have been utilized in combination with Yu, let alone to produce the presently claimed invention. As discussed throughout, one of ordinary skill in the art would not have predicted that methods for targeting noncationic, non-nucleic acid-comprising liposomes could be used to target the liposomes disclosed in Yu, and in fact, such a combination would have proceeded contrary to the excepted wisdom in the art at the time. Neither the disclosure of Xu, nor the disclosure of Sherman, are able to cure the deficiencies described above. Hence, Applicants respectfully submit that the presently claimed invention as not been rendered obvious by the combination of these references.

In view of the foregoing remarks, Applicants respectfully submit that claims 1-4, 7, 8, 12, 69 and 73-76 are not rendered obvious by the disclosures of Yu, In View of Marks, Park, Morishige, Xu and Scherman, alone, or in combination. Hence, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

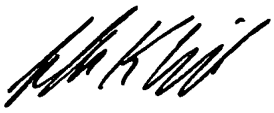
Conclusion

All of the stated grounds of rejection have been properly traversed, rendered moot or otherwise overcome. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and objections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



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Sterically Stabilized Anti-HER2 Immunoliposomes: Design and Targeting to Human Breast Cancer Cells *in Vitro*†

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Received August 26, 1996; Revised Manuscript Received November 4, 1996⊗

ABSTRACT: Liposomes (70−100 nm) of 1-palmitoyl-2-oleoylphosphatidylcholine, cholesterol, and poly(ethylene glycol) (PEG)-modified phosphatidylethanolamine (PEG-DSPE) were conjugated to Fab′ fragments of a humanized recombinant MAb against the extracellular domain of HER2/*neu* to create sterically stabilized immunoliposomes (anti-HER2 SL) as a drug carrier targeting HER2-overexpressing cancers. Conjugation employed maleimide-terminated membrane-anchored spacers of two kinds: a short spacer, providing attachment of Fab′ close to the liposome bilayer, or a long spacer, with Fab′ attachment at the distal terminus of the PEG chain. Confocal microscopy and spectrofluorometry of HER2-overexpressing breast cancer cells incubated with fluorescently labeled anti-HER2 SL prepared with either spacer showed binding of liposomes (8000−23 000 vesicles/cell) followed by endocytosis (rate constant k_e = 0.012−0.033 min^{-1}) via the coated-pit pathway, evidenced by intracellular acidification and colocalization with transferrin. Uptake of anti-HER2 immunoliposomes by breast cancer cells with low HER2 expression, or after preincubation of cells with free anti-HER2 Fab′, was less than 0.2% and 4.3%, respectively, of the uptake by HER2-overexpressing cells. Increasing PEG-DSPE content (up to 5.7 mol %) in anti-HER2-SL prepared with the short spacer decreased liposome−cell binding affinity 60−100-fold, while k_e decreased only 2-fold; however, when Fab′ fragments were conjugated via a PEG spacer, both binding afffinity and k_e were unaffected by PEG-DSPE content. Cell binding and internalization of anti-HER2 immunoliposomes increased at higher surface density of conjugated Fab′ fragments, reaching plateaus at ~40 Fab′/liposome for binding and ~10−15 Fab′/liposome for internalization. Uptake of anti-HER2 immunoliposomes correlated with the cell surface density of HER2 and significantly ($p < 0.005$) correlated with the antiproliferative effect of the targeting antibody but not with the total level of cellular HER2 expression. The results obtained were used to optimize *in vivo* preclinical studies of anti-HER2 SL loaded with antineoplastic drugs.

Antibody-based targeting is a promising approach in the development of targeted therapies for cancer (Begent, 1990; Bator & Reading, 1991). Among various antigens found on malignant cells, glycoprotein p185^{HER2}, a member of the EGFR[1] family of receptor tyrosine kinases encoded by the HER2/*neu* (c-erbB-2) protooncogene, is an attractive target for therapy. This protein is overexpressed in various cancers, including breast, lung, and ovarian carcinomas (Slamon *et al.*, 1989; Tripathy & Benz, 1992; De Potter, 1994; Molland *et al.*, 1996). Overexpression of p185^{HER2} is also unique to the malignant phenotype (Press *et al.*, 1990). A variety of p185^{HER2}-specific monoclonal antibodies have been developed (Tagliabue *et al.*, 1989; Hudziak *et al.*, 1991) and used for the delivery of conjugated toxins (Rodriguez *et al.*, 1993; Wels *et al.*, 1995), prodrug activators (Eccles *et al.*, 1994; Rodrigues *et al.*, 1995), cytotoxic lymphocyte recognition markers (Shalaby *et al.*, 1995; Wels *et al.*, 1995; Zhu *et al.*, 1995), and plasmid DNA (Fominaya & Wels, 1996) to HER2-overexpressing tumor cells in culture and in animal models.

4D5, a murine MAb directed against the extracellular domain of p185^{HER2}, binds to abundant sites on the surface of HER2-overexpressing cells and elicits a partially agonistic response, which includes receptor internalization and phosphorylation, and ultimately yields inhibition of cell prolifera-

† This work was supported by NIH Grant P50CA58207.

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[1] Abbreviations: Chol, cholesterol; DSPE, 1,2-distearoyl-3-*sn*-glycerophosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, EGF receptor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES-buffered saline (20 mM HEPES-Na, 144 mM NaCl, pH 7.2); HPTS, 8-hydroxypyrenetrisulfonic acid trisodium salt; MMC, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; MP, *β*-(*N*-maleimido)propionyl; MP-PEG-SC, *ω*-MP-amidopoly(oxyethylene)-*α*-succinimidyl carboxylate; PBS, phosphate-buffered saline (KH_2PO_4, 0.2 g/L; $Na_2HPO_4 \cdot 7H_2O$, 2.16 g/L; KCl, 0.2 g/L; NaCl, 8.0 g/L; pH 7.4); PE, phosphatidylethanolamine; PEG, poly(ethylene glycol); PEG-DSPE, *N*-[*ω*-methoxypoly(oxyethylene)-*α*-carbonyl]-DSPE; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-glycerophosphocholine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; rhuMAbHER2, recombinant humanized anti-p185^{HER2} monoclonal antibody; SL, sterically stabilized liposomes.

S0006-2960(96)02148-4 CCC: $14.00

tion (Hudziak *et al.*, 1989). A recombinant, fully humanized version of 4D5 (rhuMAbHER2) has antiproliferative activity against HER2-overexpressing cancers *in vitro* and *in vivo* (Carter *et al.*, 1992). We have recently described a liposomal drug delivery system targeted to HER2-overexpressing cancer cells by attachment of Fab′ fragments of rhuMAb-HER2 to the membrane of small unilamellar liposomes using an amphiphilic maleimide-terminated anchor MMC-PE (Park *et al.*, 1995). Such anti-HER2 immunoliposomes bind to the cell surface and become avidly endocytosed by the target cells. Loading of anti-HER2 immunoliposomes with doxorubicin provides superior toxicity against HER2-overexpressing breast cancer cells, in comparison to nontargeted liposomal doxorubicin, while causing minimal toxicity against nonmalignant cells *in vitro*.

Liposomal anticancer pharmaceuticals benefit from "steric stabilization" achieved by conjugation of poly(ethylene glycol) to the liposome surface (Woodle & Lasic, 1992; Lasic & Papahadjopoulos, 1995). Sterically stabilized liposomes have lower reticuloendothelial uptake, prolonged circulation time in the blood, and higher accumulation in tumors (Papahadjopoulos *et al.*, 1991; Papahadjopoulos & Gabizon, 1995). However, our previous study showed that surface-grafted PEG (M_r 2000) at more than 1.3 mol % of total lipid (2% of total phospholipid) substantially reduced the uptake and cytotoxicity of doxorubicin-loaded anti-HER2 immunoliposomes in the cultures of target cells (Park *et al.*, 1995).

In the present work, we explore the hypothesis that the mode of anti-p185^{HER2} Fab′ conjugation to the liposome in relationship to membrane-anchored amphipathic PEG affects the functional interaction between the anti-HER2 immunoliposome and the target cell. We analyze the process of Fab′−immunoliposome binding and internalization into HER2-overexpressing target cells, with regard to Fab′ density and level of HER2 overexpression. On the basis of these studies, we offer a new, modified design of sterically stabilized anti-HER2 immunoliposomes that exhibit high levels of selective internalization by HER2-overexpressing breast cancer cells.

MATERIALS AND METHODS

Materials

Phospholipids and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (egg transphosphatidylated) were obtained from Avanti Polar Lipids (Alabaster, AL); cholesterol was obtained from Calbiochem (San Diego, CA); 8-hydroxypyrenetrisulfonic acid trisodium salt was obtained from Molecular Probes (Eugene, OR). MMC-DSPE was synthesized from *N*-succinimidyl-4-(*N*′-maleimidomethyl)-cyclohexane-1-carboxylate (Sigma, St. Louis, MO) and DSPE according to Martin and Papahadjopoulos (1982). ω-*N*-(β-Maleimidopropionyl)aminopoly(oxyethylene)-α-*N*-succinimidyl carbonate derived from PEG with M_r 2000 was from Shearwater Polymers (Huntville, AL); organic solvents (HPLC grade) and silica-60 TLC plates were from Fisher (Pittburgh, PA); other chemicals were of reagent purity from Sigma (St. Louis, Mo). Methoxy-PEG-DSPE and amino-PEG-DSPE were prepared from PEG (M_r 1900) according to Zalipsky (1993) and Zalipsky *et al.* (1994). Fab′ fragments of rhuMAbHER2 were prepared as described (Park *et al.*, 1995). Cell lines were obtained and cultured as previously described (Lewis *et al.*, 1993; Scott *et al.*, 1993; Park *et al.*, 1995). Media were supplied by the UCSF Cell Culture Facility.

Methods

Synthesis of Maleimido-PEG-DSPE. Poly(ethylene glycol) derivatives of phosphatidylethanolamine modified with a maleimide group at the distal terminus of the poly(ethylene glycol) chain were synthesized by two methods (Figure 1). In the first method, amino-PEG-DSPE (Zalipsky *et al.*, 1994) (500 mg, 0.18 mmol) and *N*-succinimidyl-3-(*N*-maleimido)-propionate (62.6 mg, 24 mmol) were dissolved in CH_2Cl_2 (3 mL) and DMF (0.75 μL) followed by triethylamine (76 μL, 0.54 mmol). After 15 min TLC ($CHCl_3$:CH_3OH:H_2O = 90:18:2, visualized with ninhydrin spray) showed that the reaction was complete. The product mixture was purified on the silica gel column eluted with a stepwise gradient of methanol (0−14%) in chloroform. The pure product-containing fractions (eluted in $CHCl_3$:CH_3OH = 88:12) were combined and evaporated and then further dried in vacuo over P_2O_5, yielding MP-PEG-DSPE as a white solid (202.8 mg, 44.2%). ^{1}H NMR (CD_3OD): δ 0.88 (multiplet, 6H), 1.26 (s, CH_2, 56H), 1.58 (br m, CH_2CH_2C=O, 4H), 2.31 (2 t, CH_2C=O, 4H), 2.48 (t, MP-CH_2CH_2C=O), 3.53 (t, CH_2N, 2H), 3.63 (s, PEG, 180H), 3.88 and 3.98 (q and t, CH_2PO_4-CH_2, 4H), 4.20 (t, CH_2O_2CN, 2H), 4.17 and 4.39 (2 dd, OCH_2CHCH_2OP, 2H), 5.2 (m, $PO_4CH_2CHCH_2O$, 1H), 6.69 (s, maleimide, 4H). In the similar way, MMC-PEG-DSPE was prepared from *N*-succinimidyl 4-(*N*′-maleimidomethyl)-cyclohexane-1-carboxylate with 70% yield (DSPE). In the second method, MP-PEG-SC (100 mg, 50 μmol) was reacted with DSPE (35 mg, 46 μmol) and triethylamine (13.6 μL, 100 μmol) in 2 mL of chloroform for 4 h at 45 °C. The product was purified by chromatography on silica gel as above and obtained with the yield of 74% (DSPE). All products revealed single spots on TLC (silica 60; $CHCl_3$:MeOH:H_2O = 65:25:4) after visualization by iodine vapor and molybdate spray. After solubilization of the products in the presence of 1% Triton X-100, maleimide groups were assayed according to Sedlack and Lindsay (1968); phosphate groups were quantified according to Morrison (1964). The ratio of maleimide to phosphate groups was found to be 0.90−1.0 (theory, 1.0).

Liposome Preparation. Liposomes were prepared from POPC and Chol (3:2 molar ratio) by lipid film hydration, followed by membrane extrusion (Olson *et al.*, 1979). When indicated, mPEG-DSPE was included into the lipid mixture at an amount of 0.6−5.7 mol % of total lipid. For antibody conjugation, the maleimide-terminated phospholipid derivative was included at 1.2 mol % of total lipid. When maleimido-PEG-DSPE was used as a linker, PEG-DSPE content was calculated as the total of maleimide-terminated and methoxy-terminated PEG-DSPE. For confocal microscopy studies, 0.1 mol % of fluorescently labeled phospholipid (Rho-PE) was added to the lipid mixture. Multilamellar liposomes formed by shaking of the dry lipid film in HBS or in solution containing 35 mM HPTS, pH 7.0, adjusted to an osmolarity of 280 mOsm/L with NaCl, were extruded 10 times at room temperature through two stacked 0.1 μm polycarbonate membranes. When appropriate, unentrapped HPTS was removed by gel filtration on Sephadex G-25, and the liposomes were stored at approximately 5 mM phospholipid in HEPES-buffered saline at 4 °C. Liposome size was measured by dynamic laser light scattering (Coulter N4 particle size analyzer); liposome concentration was deter-

mined by phospholipid phosphate assay (Morrison, 1964) and expressed as molar concentration of liposome phospholipid.

Conjugation of Anti-HER2 Fab' to Liposomes. Fab' fragments of rhuMAbHER2 were added to the liposomes (7−10 mM) containing maleimide-terminated linker in HEPES-buffered saline at the ratio of 0.3 mg of protein/mL, and after pH adjustment to 7.3−7.4 (NaOH) the mixture was incubated overnight at ambient temperature under argon. Excess maleimide groups were quenched by incubation with 2 mM β-mercaptoethanol for 30 min; β-mercaptoethanol and unconjugated antibody were removed by gel chromatography on Sepharose 4B (eluant HBS). Immunoliposomes were collected in the void volume fraction, sterilized by passage through a 0.2 μm sterile filter, and stored at 4 °C. The amount of conjugated Fab' in the immunoliposomes was determined by dye binding assay (Bio-Rad) and converted into the number of Fab' per liposome, assuming a liposome size of 100 nm, an average area per phospholipid molecule of 75 Å^2, which gives 80 000 phospholipid molecules/vesicle (Marsh, 1990), and a molecular weight of the rhuMAbHER2 Fab' fragment equal to 46 000. To prepare immunoliposomes with various amounts of conjugated antibody, the initial ratio of Fab' to maleimide-activated liposomes was varied.

Confocal Microscopy of the Cellular Uptake of Anti-HER2 Immunoliposomes. SK-BR-3 and MCF-7 cells were grown on coverslips to subconfluency. Cells were coincubated with Rho-PE-labeled anti-HER2 SL and FITC-labeled transferrin at saturating concentrations in serum-supplemented growth media at 37 °C for varying time periods, washed extensively with PBS, mounted in glycerol, and observed with a Molecular Dynamics MultiProbe 2001 confocal microscope.

Spectrofluorometric Measurement of Immunoliposome Uptake by the Cells. Simultaneous quantitation of cell surface-bound and endocytosed liposomes was performed according to Straubinger *et al.* (1990). Cells grown to subconfluency were incubated at 37 or 4 °C with HPTS-loaded anti-HER2 immunoliposomes diluted to 0.025 mM phospholipid in 10% serum-supplemented growth medium. For comparison of the liposome uptake by cells with different HER2 expression, the cells were incubated for 2 h with 0.1 mM anti-HER2 immunoliposomes in the growth media at 37 °C. The cells were then rinsed four times with ice-cold HEPES-buffered saline and harvested in 5 mM EDTA/PBS, and the fluorescence excitation spectra of the cell suspensions in the range of 400−500 nm were recorded using a SPEX Fluorolog 2 photon counting spectrofluorometer (SPEX Industries, Edison, NJ) at an emission wavelength of 512 nm. After subtraction of autofluorescence, the fluorescence intensities at excitation wavelengths of 454 nm (I_{454}) and 413 nm (I_{413}) were determined. The total amount of cell-associated liposomes ("liposome uptake") was determined from I_{413} by comparison to the fluorescence of liposome standards. The proportion of liposomes bound to the cell surface (neutral pH compartment) ($[L]_s$) was found from the formula:

$$\% [L]_s = (r - r_0)/(r_{100} - r_0) \times 100 \quad (1)$$

where $r = I_{454}/I_{413}$ of the sample, $r_{100} = I_{454}/I_{413}$ of the liposomes in the cell harvesting buffer (pH 7.4), and $r_0 = I_{454}/I_{413}$ of the endocytosed liposomes. The latter ratio was determined from the fluorescent spectra of cells incubated with HPTS-loaded anti-HER2 immunoliposomes for 2 h and postincubated in the liposome-free medium for 3 h, which afforded complete endocytosis of the cell-associated liposomes (Park *et al.*, 1995). The amounts of cell surface-bound and endocytosed liposomes, normalized to the cell concentrations, were plotted vs incubation time, and the kinetic curves obtained were used to characterize the uptake of liposomes by the cells. Internalization of the liposomes was characterized by the first-order endocytosis rate constant (k_e) derived from the above kinetic curves using the formula (Lee *et al.*, 1993):

$$k_e = (d[L]_i/dt)_{ss}/[L]_{s,ss} \quad (2)$$

where $[L]_i$ is the amount of internalized liposomes (per unit cell concentration), $[L]_s$ is the amount of cell surface-bound liposomes, $(d[L]_i/dt)_{ss}$ is the liposome uptake rate at the steady state determined as the slope of the curve $[L]_i$ vs time at the steady-state time point ($d[L]_s/dt = 0$), and $[L]_{s,ss}$ is the steady-state concentration of cell surface-bound liposomes. To assess the effect of free antibody on the liposome−cell interactions, free Fab' fragments of rhuMAbHER2 were added to the cells 30 min prior to addition of the liposomes.

Immunoliposome Binding to SK-BR-3 Cells. The plated cells were incubated with serial dilutions of HPTS-loaded immunoliposomes (0.2−500 μM of liposomal phospholipid) in the cell growth medium for 7 h under gentle agitation. To avoid endocytosis of the bound liposomes, the incubation was carried out at 4 °C. After incubation, the cells were washed and harvested, and the amount of cell-associated liposomes was determined by fluorometry as described above. The dissociation constant of the liposome−cell complex (K_{diss}) and the maximum liposome−cell binding ($[L]_{s,max}$) were determined as best fit parameters of the equation:

$$[L]_s = [L]_{s,max}[L]_0/(K_{diss} + [L]_0) \quad (3)$$

where $[L]_s$ is the amount of cell-bound liposomes per 10^6 cells and $[L]_0$ is the concentration of liposomes in the incubation medium. Since the amount of cell-associated liposomes was always less than 2% of the total, the concentration of free liposomes in the incubation medium at equilibrium was assumed to be equal to the initial liposome concentration, and the applicability of eq 3 was warranted. Best fit parameters were calculated using Sigma Plot 4.1 software (Jandel Scientific, Corte Madera, CA).

Statistical Methods. All experimental points are the mean of at least three parallel runs; unless indicated otherwise, standard errors were less than 8%. Standard errors for binding and kinetic parameters, as well as correlation coefficients for linear regressions, were obtained as part of computational routines used by Sigma Plot 4.1. Probabilities of null hypothesis for correlation were calculated using Student's *t*-test.

RESULTS

We have used two types of membrane-bound maleimide-terminated linkers for the conjugation of anti-HER2 Fab' fragments to liposomes utilizing the unique free thiol group in the Fab' hinge region. In the first type, a linker with a short spacer group, MMC-DSPE, provided attachment of the antibody fragments close to the liposome bilayer (Martin &

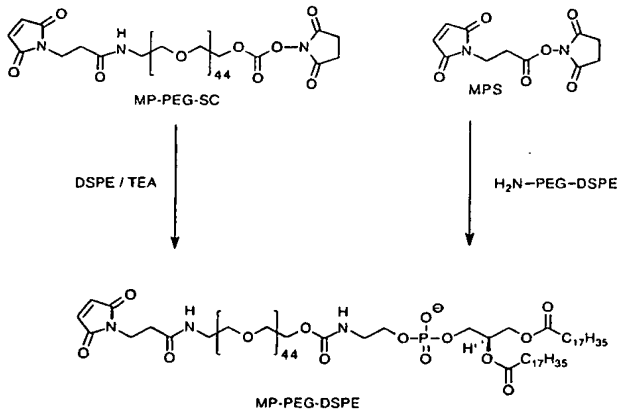

FIGURE 1: Synthesis of PEG-DSPE derivatives with the maleimide function at the end of the poly(ethylene glycol) chain.

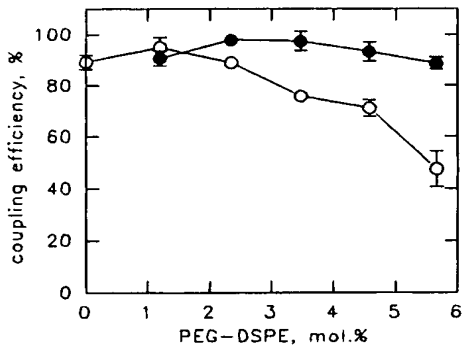

FIGURE 2: Coupling efficiency of Fab′ to maleimide-activated SL with various amounts of PEG-PE. Conjugation linker: MMC-PE (short spacer) (○); MP-PEG (M_r 2000)-DSPE (long spacer) (●). Coupling efficiency: amount of liposome-conjugated protein as percent of total protein taken for conjugation.

Papahadjopoulos, 1982). The use of the *N*-(maleimidomethyl)cyclohexyl group allowed practically quantitative conjugation of anti-HER2 to the liposomes, limited only by the proportion of reduced thiol groups in the antibody preparation. However, surface-grafted PEG chains (M_r = 2000) were likely to disturb the interaction between conjugated Fab′ and its target antigen (Klibanov *et al.*, 1990; Park *et al.*, 1995). Therefore, in the second approach, Fab′ were conjugated to the termini of PEG chains. For this approach, we synthesized two linkers, MMC-PEG-DSPE and MP-PEG-DSPE, bearing maleimide groups at the distal end of the PEG chain attached to a hydrophobic membrane anchor DSPE. Two methods were used (Figure 1): coupling of *N*-succinimidyl maleimidopropionate to the amino group of H_2N-PEG-DSPE prepared as described earlier (Zalipsky *et al.*, 1994) or coupling of DSPE to a heterobifunctional PEG derivative, MP-PEG-SC, constructed in analogy to similar SC derivatives of the polymer (Zalipsky, 1993; Zalipsky *et al.*, 1994; Allen *et al.*, 1995). Both methods were equally effective and resulted in comparable yields of the maleimido-PEG-DSPE. MMC derivatives were formed by the first method using the *N*-hydroxysuccinimidyl ester of 4-(*N*′-maleimidomethyl)cyclohexanecarboxylic acid. Both MP and MMC end-group functionalized PEG-DSPE derivatives proved equally effective for preparation of immunoliposomes. In the liposomes containing up to 2.3 mol % of PEG-DSPE, maleimido-PEG-DSPE derivatives provided conjugation with the same efficiency as their counterparts without the long PEG spacer (Figure 2); however, when the content of PEG-DSPE increased to 3.5 mol % and more, which corresponds to the transition from "mushroom" to "brush" conformation of the liposome-grafted PEG (Kenworthy *et al.*, 1995b), the conjugation efficiency of the short-chain maleimide linker decreased, while in the case of PEG-spacer linkers, the conjugation was practically quantitative up to the maximum PEG-DSPE content studied (5.7 mol %) (Figure 2). At the chosen protein/lipid ratio, the conjugation resulted in 60−80 Fab′/liposome, while by increasing the Fab′/lipid ratio in the conjugation mixture this number could be increased to 100−120 Fab′/liposome without any loss in the coupling efficiency. HPTS-loaded liposomes released less than 1% of entrapped solute during Fab′ conjugation and subsequent incubation at 37 °C in the presence of 10% serum. Immunoliposomes were stored for several months at 4 °C in the HBS buffer without any detectable leakage of entrapped HPTS or reduction in uptake by HER2-overexpressing cells.

Confocal fluorescence microscopy studies were performed to visualize the internalization and subsequent intracellular distribution of immunoliposomes. Immunoliposomes containing 3.82 mol % of PEG-DSPE, conjugated to anti-HER2 Fab′ via the MMC-PEG-DSPE derivative and labeled with Rho-PE, were incubated with SK-BR-3 breast cancer cells, which overexpress HER2 (10^6 molecules/cell), and with MCF-7 breast cancer cells which have very low or basal levels of HER2 expression (10^4 receptors/cell) (Lewis *et al.*, 1993). As an additional control, cells were coincubated with FITC-labeled transferrin, a ligand which undergoes rapid receptor-mediated endocytosis (Vidal *et al.*, 1987) in both cell lines. After 10 min incubation immunoliposomes remained largely at or near the cell surface accompanied by some cytoplasmic localization (Figure 3A), compared with the rapid endocytosis of transferrin (Figure 3A). By 30 min, immunoliposomes were observed distributed throughout the cytoplasm and extensively colocalized with transferrin in endocytic vesicles as evident from superimposed images of rhodamine and fluorescein fluorescence (Figure 3B). The specificity of immunoliposome uptake was confirmed by preincubation of SK-BR-3 cells with rhuMAbHER2 at 10-fold excess over immunoliposomes; preincubation was able to block immunoliposome uptake but not that of transferrin. In addition, MCF-7 cells similarly incubated with immunoliposomes and transferrin showed uptake of transferrin but no detectable uptake of immunoliposomes (Figure 3C).

To quantitate the cellular uptake, the liposomes were prepared with encapsulated pH-sensitive, membrane-impermeable fluorescent marker (HPTS). Entry of HPTS-loaded liposomes into the acidic environment of endocytic vesicles causes a sharp decrease in fluorescence at λ_{ex} 454 nm while its fluorescence at the isosbestic point (λ_{ex} 413 nm) remains unchanged, therefore allowing simultaneous quantitation of total vs endosome-localized cell-associated liposomes (Straubinger *et al.*, 1990). Upon incubation of HPTS-loaded anti-HER2 immunoliposomes with SK-BR-3 cells, we observed a progressive decrease in the I_{454}/I_{413} fluorescence ratio, indicating the transfer of cell-associated liposomes from the neutral to low pH compartment (Figure 4). As a control, subsequent incubation with a lysosomotropic agent (NH_4-Cl) was performed and, as expected, resulted in an increase of pH in the cellular compartments harboring anti-HER2 immunoliposomes. This effect could be reversed by removal

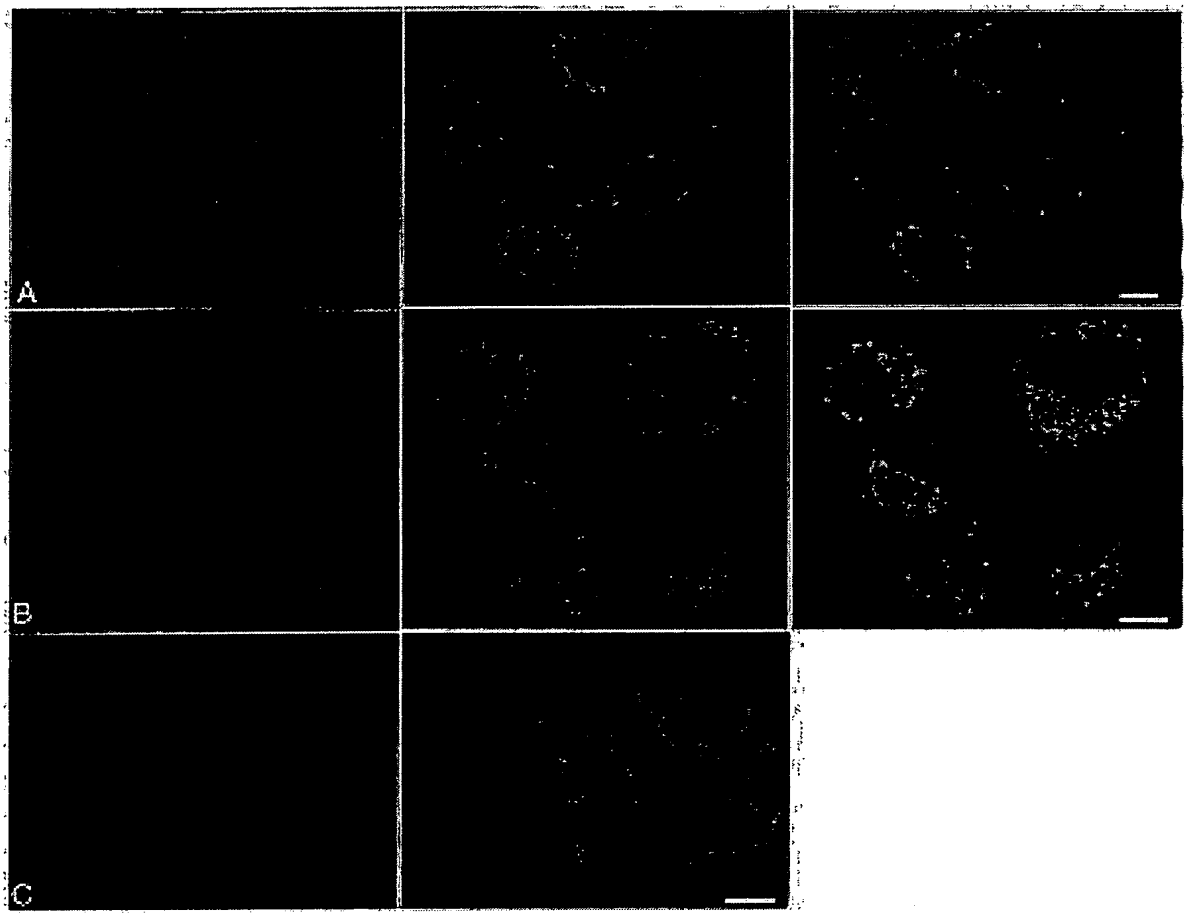

FIGURE 3: Confocal microscopy of cells coincubated at 37 °C with rhodamine-labeled anti-HER2 immunoliposomes (3.5 mol % PEG-DSPE, Fab′ conjugated via PEG spacer) and fluorescein-labeled transferrin: left column, liposomes (red); central column, transferrin (green); right column, superimposed images. SK-BR-3 cells: incubation time 10 min (A) and 30 min (B). MCF-7 cells: incubation time 30 min (C). Scale bar 10 = μm.

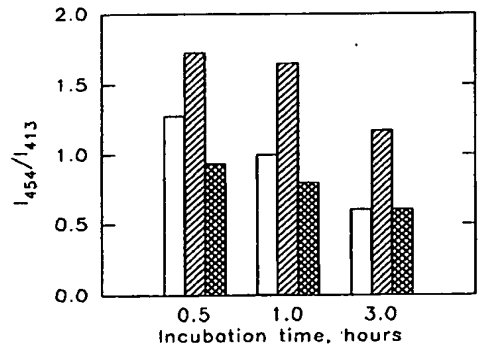

FIGURE 4: Ratio of HPTS fluorescence at excitation wavelengths 454 and 413 nm (I_{454}/I_{413}) after incubation of HPTS-loaded anti-HER2 immunoliposomes with SK-BR-3 cells (100 μM liposome phospholipid in cell growth medium at 37 °C). At indicated times, cells were washed with PBS and treated with 5 mM NH_4Cl in PBS for 20 min. Bars: open, cells before treatment with NH_4Cl; dashed, cells after NH_4Cl treatment; cross-hatched, cells after removal of NH_4Cl and 20 min postincubation in fresh medium.

of NH_4Cl (Figure 4). These data indicated that cell-associated liposomes were indeed localized in endosomes and lysosomes. A 30-min preincubation of SK-BR-3 cells with free rhuMAbHER2 Fab′ at 10-fold molar concentration relative to liposome-conjugated Fab′ reduced the uptake of anti-HER2 immunoliposomes (25 μM phospholipid) to 4.3% of its value without free Fab′. Finally, the cellular uptake of SL without conjugated anti-HER2 Fab′ (but with the thiol-quenched maleimide-terminated linker) even after prolonged incubation at the highest studied concentration (1 mM) was less than 0.01 nmol/10^6 cells, or less than 1% of the uptake of anti-HER2 immunoliposomes (Table 3).

To assess the effect of Fab′ density on immunoliposome uptake by target cells, HPTS-loaded anti-HER2 immunoliposomes containing a range of 6−81 Fab′/liposome were incubated with SK-BR-3 cells for 2−6 h. The amount of total cell-associated immunoliposomes increased with increasing density of liposome-conjugated anti-HER2 Fab′ until the plateau was reached at about 40 Fab′/liposome, or one Fab′ per 1000 phospholipid molecules in the outer leaflet of the liposome bilayer (Figure 5A). This result suggested that there is no need to increase the amount of liposome-conjugated protein over 40 Fab′/vesicle in order to achieve efficient delivery of anti-HER2 immunoliposomes into target (HER2-overexpressing) cells. An even lower number of liposome-conjugated Fab′ fragments was required to ensure internalization of the surface-bound anti-HER2 immunoliposomes. Already at the lowest Fab′ density studied (~6 Fab′/liposome, or one Fab′ per 8000 phospholipids in the outer leaflet) 55−60% of the cell-associated anti-HER2 immunoliposomes were endocytosed, and this value reached 80−95% for the liposomes bearing more than 10−15 Fab′/

Table 1: Uptake of Anti-HER2 Immunoliposomes by Cancer Cells with Various Levels of p185^{HER2} Expression

cell line[a]	total uptake, nmol of PL/mg of cell protein	% endocytosed	p185^{HER2} expression[b] total[c]	p185^{HER2} expression[b] surface[d]	cell proliferation, anti-HER2 muMAb (4D5) treated vs untreated cells,[b] %
SK-BR-3	7.21 ± 0.45[e]	88 ± 1.4	917	33.0	33
MDA-MB-453	6.52 ± 0.22	80.8 ± 0.7	43.7	16.7	61
BT-474	4.47 ± 0.21	90.4 ± 1.1	548	25.0	27
MCF-7	<0.01	0	7.27	1.2	101
MCF-7−18	4.25 ± 0.17	66.2 ± 1.9	NA	NA	NA
SK-OV-3	0.837 ± 0.096	72 ± 2.7	537	16.7	77
MKN-7	0.236 ± 0.021	13.4 ± 7.3	194	16.7	99

[a] Cells: SK-BR-3, BT-474, MDA-MB-453, MCF-7, breast carcinomas; MCF-7−18, MCF-7 stably transfected with HER2/*neu*; SK-OV-3, ovarian carcinoma; MKN-7, gastric carcinoma. [b] From Lewis *et al.* (1993). [c] ng/mg of cell protein. NA, data not available. [d] Flow cytometry using muMAb (4D5) as a marker; relative to normal mammary epithelial cells (line 184). [e] Mean ± SE.

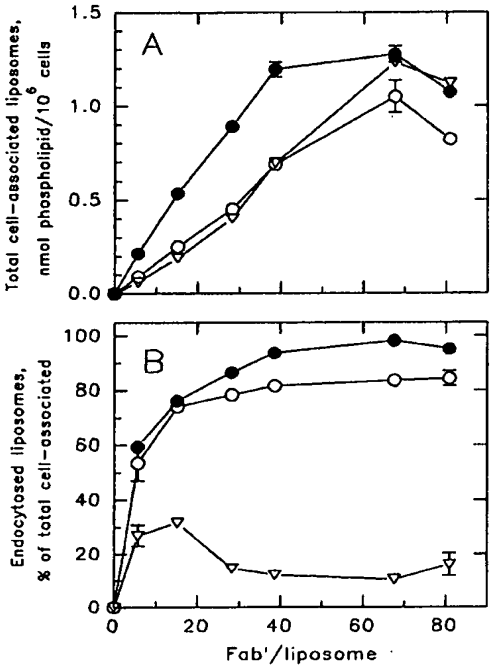

FIGURE 5: Effect of the density of liposome-conjugated anti-HER2 Fab′ on the total uptake and internalization of anti-HER2 immunoliposomes by SK-BR-3 cells: (A) total uptake; (B) endocytosed liposomes as percent of total uptake. Incubation: 2 h at 37 °C (○); 6 h at 37 °C (●); 2 h at 4 °C (▽). Liposome concentration: 25 μM of liposome phospholipid.

vesicle (Figure 5B), suggesting that liposome binding to the cells was the factor determining the extent of liposome uptake. Indeed, when the incubation was carried out at 4 °C, the total amount of cell-associated liposomes did not differ significantly from that at 37 °C, while the fraction of endocytosed liposomes was greatly reduced (Figure 5A).

Breast carcinomas, as well as other cancers, display a variety of HER2 expression levels ranging from less than 10^4 to 10^6 molecules/cell. In order to develop criteria for the future clinical use of HER2-targeted liposomal pharmaceuticals, it was of interest to study the effect of HER2 expression level on the uptake of anti-HER2 immunoliposomes. Table 1 shows the uptake (i.e., total cell-associated amount), as well as the percent of endocytosed anti-HER2 immunoliposomes (81 Fab′/vesicle; no PEG-DSPE) by cell lines expressing different levels of HER2. All four HER2-overexpressing breast cancer cell lines showed high uptake of anti-HER2 immunoliposomes, while the uptake by the cells that do not overexpress HER2 (MCF-7) was less than 1% of the value characteristic for HER2-positive cells. Two cell lines, MKN-7 (gastric carcinoma) and SKOV-3 (ovarian carcinoma), showed lower than expected uptake of anti-HER2 immunoliposomes despite high levels of HER2 expression (Lewis *et al.*, 1993). The differences in cellular uptake of immunoliposomes between these HER2-overexpressing lines could not be explained by the release of soluble extracellular domain of p185^{HER2} (which would block the antigen binding sites of liposome-conjugated Fab′) since the uptake was identical in fresh or conditioned (3 days) growth medium. We suggested that the cellular uptake of anti-HER2 immunoliposomes may be determined not only by the cellular abundance of their target antigen, HER2 protein, but also by its functional activation as a result of such binding. MKN-7, a highly HER2-positive cell line, presented an interesting case to illustrate this point. Apart from the full-length p185^{HER2}, MKN-7 cells express also a truncated version or this protein, which consists essentially of its extracellular domain, as a result of alternative splicing (Scott *et al.*, 1993). The truncated HER2 protein still provides a binding site for an anti-HER2 antibody or immunoliposome but would not functionally respond to such binding by internalization and, eventually, antiproliferative effect (Lewis *et al.*, 1993). Also, the lack of the full-size membrane-spanning domain would make the complex between immunoliposome and truncated HER2 prone to dissociation from the membrane, resulting in the even lower uptake. Indeed, the uptake of anti-HER2 immunoliposomes did not show a statistically significant correlation with the total amount of cellular p185^{HER2} recognizable by the target antibody but correlated better with the density of cell-surface expressed p185^{HER2}, and a statistically significant ($p < 0.005$) correlation was observed between the uptake of immunoliposomes and the antiproliferative activity of anti-HER2 MAb 4D5, a murine prototype of the humanized recombinant MAb used in this study (Table 2). This finding supports the hypothesis that the uptake of anti-HER2 immunoliposomes is likely to be determined not only by the density of HER2 protein but also by the ability of this protein to become functionally activated as a result of interaction with the immunoliposome.

Surface-attached PEG has been shown to interfere with the association of targeted liposomes to their cellular and molecular targets (Klibanov *et al.*, 1990; Park *et al.*, 1995). Such interference may be circumvented by conjugation of the antibody to the distal end of the PEG chains attached to the liposome surface (Hansen *et al.*, 1995; Zalipsky *et al.*, 1996). Indeed, when the short linker MMC-PE was used for preparation of anti-HER2 SL (65−90 Fab′/vesicle), inclusion of 5.7 mol % of PEG-DSPE into the liposome composition reduced their uptake (i.e., total of binding and endocytosis) by SK-BR-3 cells by an order of magnitude,

Table 2: Correlation Coefficients and Probabilities of Null Hypothesis (p) for Linear Correlation between the Uptake of Anti-HER2 Immunoliposomes, Cellular HER2 Expression, and Responsiveness of Target Cells to Anti-HER2 Antibody

uptake/parameter	total cellular p185^{HER2}	cell surface p185^{HER2}	antiproliferative effect of anti-p185^{HER2} MAb (4D5)
total	0.434 ($p = 0.398$)	0.724 ($p = 0.112$)	0.835 ($p = 0.0040$)
internalized only	0.477 ($p = 0.380$)	0.744 ($p = 0.089$)	0.863 ($p = 0.0028$)

Table 3: Binding and Internalization Parameters of Anti-HER2 Immunoliposomes with Various PEG-DSPE Content by SK-BR-3 Cells

PEG-DSPE, mol % of total lipid	conjugation spacer	Fab′/vesicle	K_{diss}, μM phospholipid[b]	$[L]_{s,max}$, nmol of phospholipid/10^6 cells[b]	k_e, min^{-1}[c]
0	MMC-DSPE	81	12.1 ± 1.7[d]	4.26 ± 0.14	0.026
1.2	MMC-DSPE	71	38.3 ± 5.7	3.70 ± 0.16	0.033
3.5	MMC-DSPE	59	1211 ± 79	15.3 ± 0.8	0.014
5.7	MMC-DSPE	35	732 ± 11	10.5 ± 1.1	0.012
1.2[a]	MP-PEG (M_r 2000)-DSPE	89	13.1 ± 2.2	1.47 ± 0.05	0.026
3.5[a]	MP-PEG (M_r 2000)-DSPE	92	9.33 ± 3.93	1.09 ± 0.10	0.018
5.7[a]	MP-PEG (M_r 2000)-DSPE	124	8.62 ± 1.72	3.02 ± 0.13	0.023

[a] Total of methoxy-PEG-DSPE and maleimido-PEG-DSPE. [b] K_{diss} and $[L]_{s,max}$ were determined at 4 °C as described in the Materials and Methods section Immunoliposome Binding to Cells. [c] k_e was determined at 37 °C using the HPTS fluorescence method as described in Materials and Methods. [d] Mean ± SE.

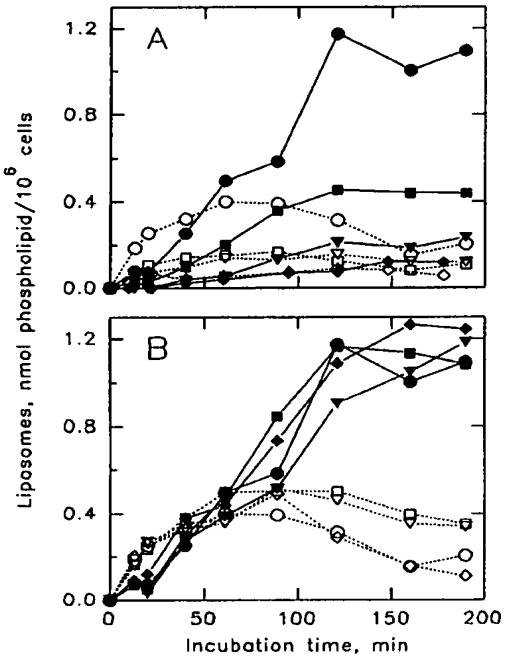

FIGURE 6: Effect of PEG-DSPE content on the uptake kinetics of anti-HER2 immunoliposomes by SK-BR-3 cells: (A) anti-HER2 Fab′ attached to the liposomes via a short spacer (MMC-DSPE); (B) Fab′ attached to PEG termini. Surface-bound liposomes: dashed line, hollow symbols. Endocytosed liposomes: solid line, filled symbols. PEG-DSPE content: none (○), 1.2 mol % (□), 3.5 mol % (▽), and 5.7 mol % (◇).

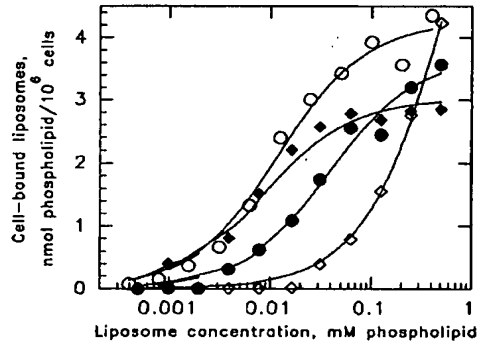

FIGURE 7: Binding of anti-HER2 immunoliposomes to SK-BR-3 cells at 4 °C. Fab′ attached via a short spacer (MMC-DSPE): no PEG-PE (○), 1.2 mol % PEG-DSPE (●), and 5.7 mol % PEG-DSPE (◇). Fab′ attached to PEG termini: 5.7 mol % PEG-DSPE (◆).

and even at 1.3 mol % of PEG-DSPE the reduction in cellular uptake was apparent (Figure 6A). On the contrary, anti-HER2-SL prepared with maleimido-PEG-DSPE as a linker showed no reduction in uptake even when the total liposome content of PEG-DSPE and maleimido-PEG-DSPE derivatives reached 5.7 mol % (Figure 6B).

Increasing PEG-DSPE content affected the cell surface binding affinity and k_e of anti-HER2-SL to HER2-overexpressing cells in a different way, dependent on whether the conjugation was *via* a short spacer or long (PEG) spacer. The binding curves (Figure 7) fit very well to the simple Langmuir-type equation of the equilibrium binding assuming all binding sites equal and independent (see eq 3 in Methods).

As shown on Table 3, the presence of PEG on the liposome surface strongly affected the binding affinity of anti-HER2-SL prepared with the short linker (K_{diss} increased 60−100-fold when PEG-DSPE contents increased from 0 to 5.7 mol %) while the internalization rate of surface-bound liposomes was much less affected (k_e decreased only 2-fold). Again, surface-grafted PEG has practically no effect on the binding affinity or internalization rate of anti-HER2-SL prepared by conjugation of Fab′ fragments to the termini of PEG chains.

DISCUSSION

Attachment of anti-HER2 Fab′ to the distal termini of PEG chains on sterically stabilized immunoliposomes was explored as a means to avoid interference from the PEG chains with its interaction with the target, p185^{HER2}, on the cell surface. This approach has been successfully applied to enzymes (Blume *et al.*, 1993), small molecules (Lee & Low, 1994), and whole antibodies (Allen *et al.*, 1994, 1995; Hansen *et al.*, 1995; Maruyama *et al.*, 1995; Zalipsky et al., 1996). However, in the latter case, the increasing number of conjugated antibody molecules led to the increasing rate of immunoliposome clearance from the circulation, possibly because of the recognition of the Fc portion of conjugated antibody by macrophages. To avoid this problem, we have used Fab′ fragments as a targeting device (Martin & Papahadjopoulos, 1982; Park *et al.*, 1995). Additionally, the

thiol group in the hinge region provided a single site for conjugation far away from the antigen binding site, which also ensured correct orientation of the conjugated Fab′ (Nässander *et al.*, 1995; Shahinian & Silvius, 1996).

Two strategies for the synthesis of maleimide-terminated PEG-DSPE derivatives (Figure 1) yielded equally active products, but the use of a heterobifunctional PEG derivative was preferred since it allowed convenient one-step synthesis from commercially available precursors, DPPE and MP-PEG-SC. The stability of the maleimide function in the chosen groups (MP and MMC) at neutral pH proved to be sufficient to ensure quantitative conjugation of Fab′ fragments bearing reactive free thiols. When a short maleimide-terminated linker (MMC-PE) was used for Fab′ conjugation to the liposomes containing PEG-DSPE, the efficiency of conjugation somewhat decreased at PEG-DSPE concentrations corresponding to the predominantly brush conformation of the polymer (Figure 2), possibly due to the hindered penetration of the Fab′ fragment through the polymer "cloud"; however, the hindrance was less pronounced than expected from the theoretical prediction by Torchilin *et al.* (1994). This observation agrees with the results of Hansen *et al.* (1995), who found no decrease in the conjugation efficiency of the thiolated whole IgG to SL containing 5 mol % PEG-DSPE and activated with the short-chain maleimide linker (maleimidophenyl)butyryl-PE.

HER2-overexpressing cells showed considerable uptake of anti-HER2 SL, reaching 8000−23 000 vesicles/cell at saturating liposome concentrations. The selectivity of the uptake was also high: uptake by the cell line with low HER2 expression was at least 2 orders of magnitude lower than by HER2-overexpressing cells (Table 1). The same selectivity was achieved in the uptake of anti-HER2 immunoliposomes, with or without PEG-DSPE, by HER2-overexpressing cells (1−6 nmol/10^6 cells) in comparison to nontargeted SL ($\leq$0.01 nmol/10^6 cells). The quenching of excess active groups following the conjugation reaction seems to be important in achieving good selectivity of targeted liposome uptake, since in the presence of free thiols or hydrazide groups at the PEG termini, sterically stabilized immunoliposomes show appreciable background binding to the cells lacking the target antigen (Allen *et al.*, 1995; Hansen *et al.*, 1995), possibly due to nonspecific covalent attachment to cell surface molecules (thiol exchange or hydrazone formation with terminal sugar residues of cell surface glycoproteins).

Both confocal microscopy and spectrofluorometry of liposome-entrapped HPTS indicated endosomal localization of internalized anti-HER2 SL. Anti-HER2-SL bound to target cells became internalized and eventually, but not immediately, colocalized with endocytosed fluorescein-labeled transferrin (Figure 3). Since transferrin is shown to enter the cells by receptor-mediated endocytosis via a coated-pit mechanism and colocalize with the antibody-stimulated HER2 receptor (Maier *et al.*, 1991), this observation suggested that anti-HER2-SL are endocytosed through the similar process, likely associated with the internalization of the HER2 receptor. The internalization rate of anti-HER2-SL was visibly slower than that of transferrin, which was consistent with the kinetic data: k_e 0.018 min^{-1} for anti-HER2 SL (Table 3) vs 0.23 min^{-1} for the transferrin−receptor complex (Douglas & King, 1988). Since ^{125}I-labeled transferrin conjugated to liposomes of comparable size was shown to be internalized by transferrin receptor-bearing cells at the same rate as free ^{125}I-labeled transferrin (Vidal *et al.*, 1987), the difference in the endocytosis rates between transferrin and anti-HER2-SL is likely to reflect the difference in the endocytosis rates between the transferrin−receptor complex and the antibody-activated HER2 receptor.

The density of conjugated Fab′ on the liposome surface and the density of HER2 receptors on the cell surface were important determinants of the liposome uptake by the cells. The increasing density of Fab′ fragments on the liposome surface did not lead, however, to an unlimited increase in the cellular uptake, reaching a plateau at about 35−40 Fab′ per 100 nm liposome, or one antigen binding site per each 1000 phospholipid molecules in the outer leaflet of the liposome bilayer (Figure 5A). These results are in agreement with the observation that binding *in vivo* of SL conjugated via the PEG spacer to the antibody (34A) specific for murine lung endothelium reaches saturation at about 30 antibodies per 90−130 nm liposome, or about 1.2 binding sites per each 1000 phospholipid molecules of the outer leaflet (Maruyama *et al.*, 1995).

Comparison of the uptake of anti-HER2 immunoliposomes by cells with different levels of p185^{HER2} expression showed that high expression of p185^{HER2} was a necessary but evidently not sufficient condition for the high cellular uptake of anti-HER2 immunoliposomes (Table 1). The cellular uptake of anti-HER2 immunoliposomes best correlated with the antiproliferative activity of the murine prototype of the targeting antibody, 4D5 (Table 2). The antiproliferative effect of anti-HER2 MAb has been linked to its ability to cause receptor internalization (Tagliabue *et al.*, 1991; Sarup *et al.*, 1991). This observation suggested that the uptake of anti-HER2 immunoliposomes is determined not only by the density of HER2 protein but also by the ability to promote endocytosis of the HER2 receptor, which also implies that endocytosis of the anti-HER2 immunoliposome is a consequence of the endocytosis of its target antigen, p185^{HER2}. Since monovalent Fab′ fragments lack the agonistic and antiproliferative activity of the whole bivalent 4D5 antibody (Sarup *et al.*, 1991), it further implies that immunoliposomes bearing conjugated anti-p185^{HER2} Fab′ fragments act as multivalent immunoligands capable of receptor stimulation similar to the bivalent antibody. Indeed, after a few hours of incubation with SKBR-3 cells about 50% of cell-associated immunoliposomes bearing as few as 6 anti-HER2 Fab′ fragments per vesicle were endocytosed, and cell-associated immunoliposomes bearing at least than 15 Fab′/vesicle were endocytosed with more than 80% efficiency (Figure 5B).

The attachment of targeting antibody to the termini of surface-grafted PEG chains, rather than in close proximity of the liposome bilayer, has been shown to improve the association of immunoliposomes with their target cells (Hansen *et al.*, 1995). However, in these studies the processes of surface binding and subsequent internalization of PEG-derivatized immunoliposomes were not discriminated, and the separate effects of surface-grafted PEG on these two processes still remained to be clarified. The use of the HPTS method (Straubinger *et al.*, 1990) allowed simultaneous quantitation of immunoliposome binding and endocytosis by HER2-overexpressing cells and provided evidence that decreased surface binding affinity is the major reason for the reduced cellular uptake of anti-HER2-SL with targeting Fab′ fragments attached proximally to the liposome bilayer. While the efficiency of endocytosis of surface-bound

anti-HER2 immunoliposomes, measured as the first-order internalization rate constant (k_i), decreased only 2-fold when the PEG-DSPE content increased from 0% to 5.7 mol %, the binding affinity of immunoliposomes decreased by almost 2 orders of magnitude (Table 3). Uptake of anti-HER2 immunoliposomes incubated with SK-BR-3 cells at a concentration (25 μM) close to K_{diss} (12 μM) reached the plateau at a Fab′ density of more than 35−40 Fab′/liposome (Figure 5); therefore, such dramatic reduction in affinity could not be explained by the lower coupling efficiency of high-PEG vs low-PEG liposomes (35 Fab′/liposome vs 81 Fab′/liposome), and in accordance with the results obtained in similar systems (Kibanov *et al.*, 1990; Torchilin *et al.*, 1995), it was attributed to the effect of surface-grafted PEG. The extension length of the grafted PEG in the brush conformation on the surface of phosphatidylcholine liposomes containing 6 mol % PEG (M_r 2000)-DSPE is approximately 6 nm (Kenworthy *et al.*, 1995a,b) while the length of the Fab′ molecule has been determined as 7 nm from X-ray crystallography data (Nezlin, 1977). Apparently, the repulsion between cell surface and liposome PEG coating decreases the free energy gain of the Fab′ binding to p185^{HER2} on the cell surface, therefore reducing the apparent affinity constant ($\Delta G° = -RT \ln K$). When the antigen binding site was located outside of the PEG coat, e.g., in the case of the Fab′ attachment to the distal termini of PEG chains, cell surface binding of immunoliposomes was not affected by PEG (Table 2).

Cell surface binding parameters of anti-HER2 immunoliposomes with fully exposed antigen binding sites, i.e., prepared in the absence of PEG-DSPE or by terminal conjugation of Fab′ to PEG chains, were in good agreement with the cell binding parameters of the muMAb 4D5, a prototype of the targeting antibody rhuMAbHER2. To allow comparison, K_{diss} (Table 3) was translated from the units of liposomal phospholipid concentration to the molar concentration units of conjugated anti-HER2 Fab′. The resulting values and maximum numbers of binding sites per cell were 9−12 nM and (0.7−4.4) × 10^6, respectively, compared to 19 nM and 2 × 10^6 for free 4D5 Fab and 6 nM and 0.93 × 10^6 for free 4D5 MAb (Sarup *et al.*, 1991). Immunoliposomes with high PEG-DSPE content and lower binding affinity to the cells tended to saturate the cellular uptake mechanism at higher levels. We may hypothesize that PEG coating reduces the lateral mobility of the liposome-conjugated Fab′ fragment and therefore reduces the number of Fab′ fragments that can become exposed at the contact surface between the liposome and the cell membrane, so that each immunoliposome endocytosis event consumes less molecules of the target antigen. However, it is likely that, for *in vivo* applications, the saturation binding will be less important than affinity, since the concentrations of targeted liposomes in the body tissues are unlikely to come close to saturation values.

Antibody binding to the cell surface proteins of the EGFR family often induces their internalization. It was of interest to compare the rates of immunoliposome internalization (k_e 0.012−0.033 min^{-1}; Table 3) with the rates of antibody-induced internalization of their target antigen. Since the endocytic rate constants for rhuMAbHER2-activated p185^{HER2} were unavailable, the comparison was made with Neu protein, a rat homolog of human p185^{HER2}. Using the data of Gilboa *et al.* (1995), we have estimated that the wild-type Neu activated by a bivalent agonistic antibody, as well as Neu*, a constitutively dimerized, permanently activated point mutant of Neu, expressed in NIH 3T3 cells, was endocytosed with k_e 0.024 min^{-1}, while Neu bound to nonagonistic Fab fragments of the same antibody had k_e 0.013 min^{-1}. The latter value is practically equal to that observed by us for anti-HER2 SL coated with PEG in brush conformation and bearing anti-HER2 Fab′ attached through a short spacer (Table 3, ≥3.5 mol % PEG-DSPE). Such twofold decrease in k_e, compared to that of liposomes with fully exposed Fab′, may be caused by PEG chains creating repulsion between HER2 molecules held by the liposome-attached Fab′, therefore preventing HER2 dimerization necessary to promote endocytosis. Comparison of the kinetic data, together with the confocal microscopy data and the strong correlation between immunoliposome uptake and agonistic action of the targeting antibody, leads us to conclude that the endocytosis of anti-HER2 immunoliposomes is strongly associated with the endocytosis of their target antigen, p185^{HER2}. Binding of immunoliposomes to their target cells is not always followed by the liposome internalization (Leserman & Machy, 1987). Goren et al. (1996) found that anti-HER2-SL constructed using a different targeting antibody (N-12A5), as well as a different conjugation method, selectively bind to HER2-positive human gastric carcinoma cancer cells (N-87) but do not increase the drug cytotoxicity *in vitro*, or its antitumor effect *in vivo*, compared to nontargeted doxorubicin-loaded SL, probably due to lack of cell internalization. Therefore, appropriate choice of the targeting antibody, as well as of the target antigen, may be crucial for successful development of pharmaceutical immunoliposomes capable of intracellular drug delivery.

We have described sterically stabilized immunoliposomes which efficiently bind to the cancer cells overexpressing p185^{HER2} oncoprotein and enter cells through endocytosis. The specificity of binding was achieved by conjugation of a Fab′ fragment of the recombinant humanized antibody against extracellular domain of HER2, while steric stabilization of the liposomes was provided by an amphiphilic poly(ethylene glycol) derivative PEG-DSPE. To prevent PEG chains from interferring with the antibody−antigen interaction, the target-specific antibody fragments were conjugated to the distal termini of liposome-grafted PEG chains modified with maleimide groups; free thiol groups in the area of Fab′ corresponding to the antibody hinge region were used for conjugation. The resulting sterically stabilized immunoliposomes showed unperturbed binding and internalization by HER2 overexpressing cancer cells in cell culture even at high PEG-DSPE content.

Recent advances in liposome research, including development of sterically stabilized liposomes, increased attention to liposomes as delivery vehicles for cancer treatment drugs (Lasic & Papahadjopoulos, 1995). Among the advantages offered by sterically stabilized drug-loaded liposomes are improved plasma pharmacokinetics, higher storage stability, higher tumor localization of the drug, and improved therapeutic index (Papahadjopoulos *et al.*, 1991; Gabizon, 1992; Lasic & Martin, 1995). Anticancer drugs such as doxorubicin can be stably and efficiently loaded into sterically stabilized anti-HER2 immunoliposomes as well (Park *et al.*, 1995). Preliminary results indicated that the described design of anti-HER2-SL results in the decreased blood clearance similar to that of unmodified SL and to higher efficacy of such HER2-targeted cytostatic SL, compared to their nontargeted counterparts, for the growth inhibition of HER2-

overexpressing breast cancer xenografts in nude mice (Park *et al.*, 1996). Targeting directed by an anti-p185^{HER2} antibody which endows sterically stabilized liposomes with the capability of carrying their drug load inside the target cancer cell will add a new dimension to the technology of liposomal drug delivery.

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BI962148U

Liposomes

Rational Design

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MARCEL DEKKER, INC. NEW YORK · BASEL

Library of Congress Cataloging-in-Publication Data

Liposomes : rational design / edited by Andrew S. Janoff
p. cm.
Includes bibliographical references and index.
ISBN 0-8247-0225-5 (alk. paper)
1. Liposomes. 2. Drug carriers. 3 Drug targeting. I. Janoff, Andrew S.
[DNLM: 1. Liposomes--pharmacology. QU 93 L7656 1999]
RS210.L55 1999
615'.7--dc21
DNLM/DLC
for Library of Congress 98-46309
CIP

Cover illustration: Megan E. Janoff

This book is printed on acid-free paper.

Headquarters
Marcel Dekker, Inc.
270 Madison Avenue, New York, NY 10016
tel: 212-696-9000; fax: 212-685-4540

Eastern Hemisphere Distribution
Marcel Dekker AG
Hutgasse 4, Postfach 812, CH-4001 Basel, Switzerland
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World Wide Web
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Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

PRINTED IN THE UNITED STATES OF AMERICA

4

Functional Pleomorphism of Liposomal Gene Delivery Vectors

Lipoplex and Lipopolyplex

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I. INTRODUCTION

The prospect of correcting human disorders through gene therapy has recently created much excitement in the public, industry, and academia (1,2). Many genes capable of correcting diseased phenotypes have been identified and it is now also possible to generate engineered DNA that carry potentially therapeutic genes in sufficient quantities for clinical trials. Success of gene therapy is largely dependent on the development of suitable vectors for in vivo gene transfer. A commonly used approach is to introduce a therapeutic gene into tissue(s) or cells removed from an individual and then reintroduce the transfected cells back to the host (ex vivo protocol). However, such an approach is complicated, time-consuming, costly, and therefore, has limited clinical applications. An ideal vector should be safe, stable, easy to produce in large quantities, and capable of achieving efficient and tissue-specific gene expression when directly administrated in vivo.

A number of methods have been developed for transfecting eukaryotic cells including chemical methods (calcium phosphate precipitation, polybrene, targeted polylysine conjugates, and lipidic vectors), physical methods (microinjection, particle bombardment and electroporation), and biological methods (viral vectors). All of these methods can be potentially used for ex vivo transfection, however, some methods are apparently not suitable for in vivo gene transfer,

such as calcium phosphate precipitation, electroporation, and microinjection. The vectors currently under evaluation for clinical gene therapy are mainly viral and lipidic systems (3).

Viral vectors are replication-defective viruses with part of the viral coding sequence replaced by that of a therapeutic gene(s). Viral vectors currently examined include retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, papilloma virus, and others. Viral vectors are highly efficient in transfecting cells but suffer from a number of problems, such as immunogenicity (4), toxicity (5), difficulty in large scale production (6), and potential recombination with wild-type viruses (6). As a result of such limitations, there has been a substantial effort focused on nonviral vectors, particularly on the use of lipidic systems (7,8). Lipidic systems (liposomes, micelles, and other organized structures of lipids) are attractive due to their favorable characteristics such as biodegradability, minimal toxicity, nonimmunogenecity, ease in large scale production, and simplicity of use. Liposomes, with a variety of structures and components, can be safely administered in vivo by different routes, including iv (9), ip (10), by aerosol (11), or by direct intratissue injection (9). Furthermore, covalent attachment of a specific ligand to liposomes can improve the targeting efficiency of DNA delivery to specific cell types. DNA can either be entrapped into anionic liposomes or be complexed with cationic liposomes by charge interaction. Currently, several cationic liposomal formulations are being used clinically for the gene therapy of cystic fibrosis (12–14) and cancer (15).

It should be stated that the in vivo performance of a vector depends on the administration routes. A formulation suitable for intratracheal injection may not work when administered intravenously, and vice versa. When a direct intratissue injection method is employed, some tissues can be more efficiently transfected with naked plasmid DNA, e.g., muscle (16) and certain types of solid tumor (17). This result suggests that optimization of a vector has to be individualized according to each clinical setting. It also stresses the importance of including naked plasmid DNA as a control when the efficiency of any new vector is evaluated.

Gene therapy is a rapidly developing, highly complex field and many novel gene delivery systems are being continuously explored. It is beyond the scope of this chapter to cover all aspects of the field. The remainder of this article will focus on lipidic gene delivery systems with an emphasis on cationic liposomes and several novel supramolecular assembling systems.

II. LIPIDIC GENE DELIVERY SYSTEMS

Many different lipidic systems have been explored as vectors for gene transfer in vivo as well as in vitro, such as liposomes, micelles, emulsions, and other

organized structures of lipids. Early studies used neutral and anionic liposomes for gene delivery. Plasmid DNA is encapsulated inside the vesicles and the structure of liposomes remains unchanged. Cationic liposomes interact with DNA through charge interaction and there is an extensive lipid rearrangement during the complexation of liposomes with DNA (18–20). The resulting structure of the complexes has little resemblance to cationic liposomes. Therefore, "cationic liposome/DNA complex" is somewhat of a misnomer. However, this term is still widely used in current literature to describe cationic lipid vector. The term "lipoplex" has been recommended by several experts in the field to more clearly define the complex (21). In this chapter, the advantages and disadvantages of anionic and cationic liposomal vectors will be addressed.

The third type of lipidic vector to be described in this chapter has a unique structure composed of a polycation-condensed core coated with a lipid shell (22,23). These novel vectors are named liposome-entrapped, polycation-condensed DNA, or LPD particles. Depending on the lipids used to coat the particles, LPD particles can either be positively charged (22) or negatively charged (23) and are called LPDI and LPDII, respectively. The advantages and potential problems with each vector will be discussed. This class of vector is called "lipopolyplex" as recently recommended (21).

Section VII will introduce cationic micelles and emulsions. Compared with cationic liposome/DNA complexes, they are more stable and serum-insensitive in transfecting cells in vitro (24,25).

Recent studies have showed that a hydrophobic lipid/DNA complex can be prepared in the absence of preformed liposomes. Such a complex can be isolated in an organic phase and used as an intermediate in the preparation of well-defined particles (26–28). One example is the reconstituted chylomicron remnants recently developed in our laboratory. The reconstituted chylomicron remnants are quite stable and give a high level of gene expression upon injection from the portal vein (29). This lipidic vector and some other self-assembling systems will be addressed in Section VIII.

Finally, we will describe lipidic vector-mediated in vivo gene transfer by iv administration. Ways to overcome the serum-sensitivity of lipidic vectors, particularly cationic lipidic vectors, will also be discussed.

III. NEUTRAL AND ANIONIC LIPOSOMES

Neutral and anionic liposomes have been extensively studied as drug carrier and several liposomal drug formulations have already been used clinically for the treatment of cancer and infectious diseases (30). In contrast, these liposomes did not contribute significantly to the field of gene therapy, although conventional liposomes were used as early as the early 1980s to introduce genes to cells.

Fraley et al. used a reverse-phase evaporation method to incorporate SV40 DNA into liposomes and demonstrated gene expression in transfected cells in vitro (31). Later on, this method was also employed by Nicolau et al. (32) to incorporate a rat insulin I gene into liposomes. Low level of gene expression could be detected in rats receiving iv injection of the liposome-entrapped gene (32). Other methods to encapsulate DNA inside liposomes include Ca^{2+}-EDTA chelation (33), detergent dialysis (34), ether injection (35), and repeated freezing and thawing (36). However, due to the large hydrodynamic diameter and negative charge content of the plasmid DNA and its inefficient interaction with the conventional liposomes, the entrapment efficiency of DNA in the liposomes is very limited. Also, the nonspecific interactions between these liposomes and cells are inefficient and these liposomes do not possess an endosome-disruption mechanism, therefore, cellular uptake of DNA and its cytoplasmic release proved to be inadequate. To improve gene delivery efficiency, Wang and Huang used pH-sensitive liposomes to introduce foreign DNA to cells (37,38). pH-sensitive liposomes are vesicles that are stable at physiological pH and become destabilized at the acidic endosomal pH. A typical pH-sensitive liposome formulation is composed of dioleoylphosphatidylethanolamine (DOPE) and a pH-sensitive lipid which can be a weakly acidic amphiphile N-palmitoyl homocystein (PHC) (39), oleic acid (OA) (40), or cholesterol hemisuccinate (CHEMS) (41). Typically, liposomes are prepared at a pH greater than 8.0. In the acidic environment of endosome, the pH-sensitive lipid undergoes protonation, resulting in destabilization of liposomal membrane and cytoplasmic release of the encapsulated DNA. Therefore, pH-sensitive liposomes are advantageous over conventional anionic liposomes and neutral liposomes in that they reduce lysosomal degradation of endocytosed DNA. In a later study, Wang and Huang incorporated a targeting ligand (anti-H-$2K^k$ monoclonal antibody) to pH-sensitive liposomes in order to achieve a more efficient interaction with target cells (37,38). When a plasmid DNA containing the chloramphenicol acetyltransferase (CAT) reporter gene with a cyclic AMP (cAMP)-inducible promoter was used, cell-type-specific gene expression was found in nude mice carrying ascites tumors generated by H-$2K^k$ positive RDM-4 lymphoma cells. Moreover, in vivo gene expression could be controlled by the administration of exogenous cAMP.

These early studies with neutral or anionic liposomes demonstrated the feasibility of using liposomes as a gene delivery vehicle. Yet, owing to the technical difficulties in encapsulating sufficient amount of DNA into the vesicles, these studies were not further pursued. For the last 10 years, attention has been primarily focused on cationic liposomes. Legendre and Szoka compared the in vitro DNA delivery efficiency of pH-sensitive liposomes composed of DOPE/CHEMS and cationic liposomes composed of DOTMA/DOPE. pH-sensitive liposomes were found to be 33 to 100-fold less efficient than cationic liposomes in transfecting cells (42). Such comparison was done on the basis of the amount

of DNA added to cells. Since ligand-free, pH-sensitive liposomes do not bind to cells efficiently, another comparison should have been done on the basis of the amount of DNA taken up by the cells. It should be noted that neutral and anionic liposomes are less toxic and more compatible with biological fluids and, therefore, will be more appropriate for systemic administration. In addition, a targeting ligand can be introduced into liposomes to direct gene(s) to tissues in a cell-type-specific manner. Currently, neutral and anionic particles are being re-examined and improved in several laboratories for in vivo gene delivery, some of which will be covered later in this chapter.

IV. CATIONIC LIPOSOMES

Few positively charged lipids naturally exist in human cells. The cationic lipids synthesized in early days were detergents such as DDAB and CTAB and those were mainly used for physical studies, not for gene delivery. An early study by Fraley and Papahadjopoulos showed that positively charged, stearlamine-containing liposomes were less efficient than negatively charged liposomes in transfecting cells in vitro (43). In addition, the cationic liposomes were shown to be more toxic (43) and these studies were not continued. In the late 1980s, Felgner et al. reported highly efficient in vitro transfection using cationic DOTMA liposomes and this finding renewed interest in cationic liposome-mediated gene delivery (44). To date, numerous cationic lipids have been synthesized and tested for their transfection efficiency as well as toxicity (8). There are several advantages using cationic liposomes for gene delivery. First, unlike neutral or anionic liposomes which require entrapment of DNA inside vesicles, cationic liposomes form complexes with negatively charged DNA via charge interaction. Nearly 100% of DNA can be recovered in complexed form and there is also no strict size limitation on the DNA. Second, liposome/DNA complexes are normally prepared in such a way that the complexes are in slight excess in positive charge. This allows an efficient interaction of the positively charged complexes with the negatively charged cell membrane. Depending on the lipid composition, the complexes may also possess an endosome-disruption mechanism which facilitates cytoplasmic release of DNA after its endocytosis. Finally, complexation of cationic liposomes with DNA may help protect the DNA against physical forces and enzymatic digestion. Currently, cationic liposomes are widely employed for the transfection of eukaryotic cells in research laboratories. Several liposomal formulations have also undergone clinical evaluation as vectors for gene therapy in cancer (15) and cystic fibrosis (12–14).

A. Structure-Activity Relationships of Cationic Lipids

All cationic lipid molecules contain four different functional domains: a positively charged head group(s), a spacer of varying length, a linker bond, and a

hydrophobic anchor. Based on differences in the hydrophobic anchor, cationic lipids can be grouped into three different categories: those containing cholesterol, single-chain hydrocarbon, or double-chain hydrocarbon. Single-chain hydrocarbon-anchored lipids have a nonpolar part of small volume. These lipids form micelles and are better known as detergents. Cationic detergents such as CTAB, TTAB, and DTAB are too toxic to be used alone for transfection. Yet, when these detergents are mixed with DOPE, the toxicity is decreased significantly (45). Single-chain cationic lipids are generally less efficient than double-chain lipids in transfection and have not been used for in vivo gene transfer. Lipids currently under extensive investigation are primarily cholesterol-anchored lipids and double-chain cationic lipids. Studies on the structure-activity relationships of these lipids suggest that lipids with different anchors have different structural requirements for efficient transfection.

1. Cholesterol-anchored cationic lipids. Leventis and Silvius introduced cholesterol as the hydrocarbon core when they synthesized ChoTB and ChoSC (46). Later on, a series of cholesterol derivatives (Figure 1) were synthesized by Farhood et al. and their structure-function relationships were investigated. The parameters evaluated in the study included transfection efficiency, cellular toxicity, and inhibitory effects on protein kinase C activity (47). Conclusions from the study are as follows: (1) Cationic lipids containing nondegradable ether bonds are generally more toxic than those containing biodegradable linker bonds such as ester, amide, and carbamoyl bonds; (2) Inhibition of PKC activity by a cationic lipid is associated with its cellular toxicity and impaired transfection; (3) Tertiary amines are generally more efficient than quaternary amines in transfection; and (4) A spacer of about 3–6 atoms between the amino group and linker is optimal for activity (47). These empirical rules have led to the synthesis of a cholesterol derivative, 3β-[N-N′,N′-dimethylaminoethane)carbamoyl] cholesterol (DC-chol) (48). DC-chol has a hydrolyzable carbamoyl bond, a spacer of 3 atoms and a tertiary amino group. It is the first nonviral vector used in the clinical trial of gene therapy (15). Genzyme recently synthesized a series of multivalent cholesterol derivatives and their in vivo and in vitro activity was systemically studied (49). In vivo activity was studied by looking at gene expression in trachea and lung after intranasal administration of liposome/DNA complexes. It was found that the orientation of the polyamine headgroup in relation to the lipid anchor is critical for efficient gene transfer in vivo. The lipids which had spermine or spermidine bound to a central secondary amine, resulting in a "T" shaped configuration, were much more efficient than the lipids in which polyamine was coupled via a terminal primary amine. Substitution of cholesterol with a dihydrocholesterol greatly reduced the in vivo activity. The choice of a linker is also important for high activity in vivo. A carbamate linker was found to give the highest level of gene expression in vivo while substitution of carbamate with either an urea, amide, or amine resulted in an appreciable loss of

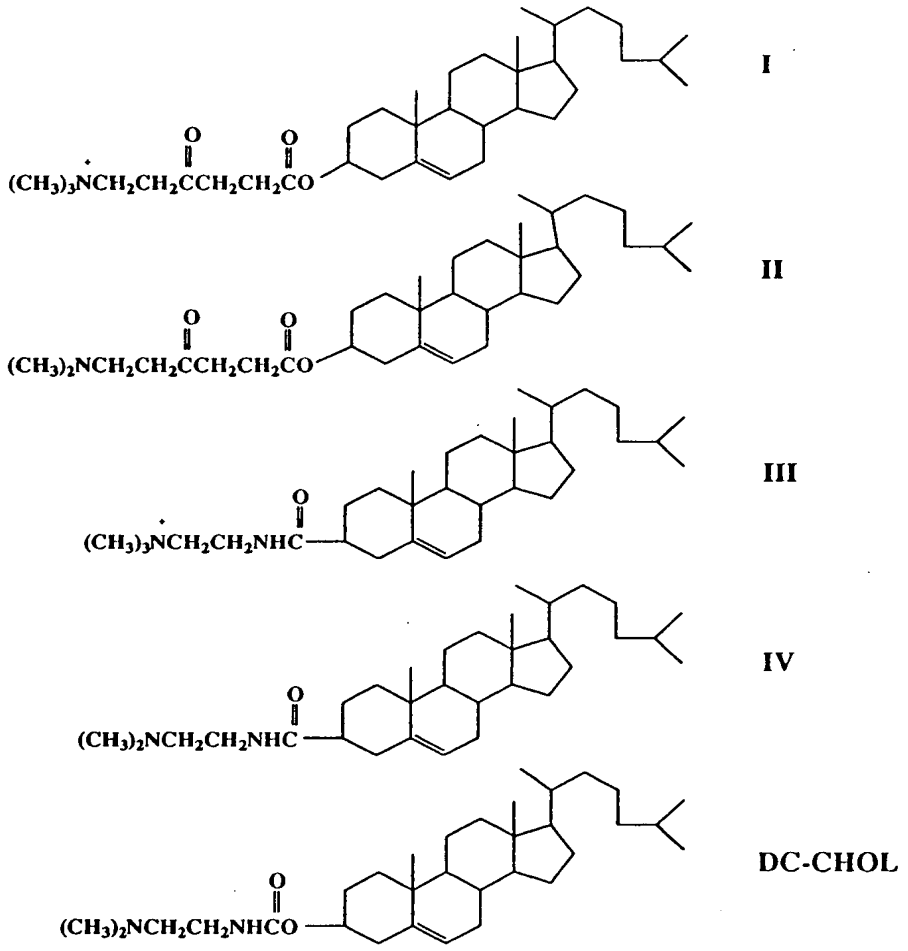
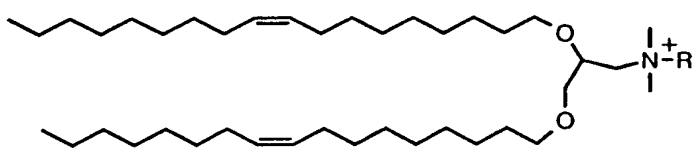


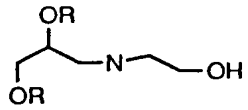
Figure 1 Cholesterol-anchored monovalent cationic lipids synthesized by Farhood et al. (From Ref. 47.)

transfection activity. Also interesting is the finding that while a cholesterol-anchor is required for maximal gene transfer in vivo, a diacyl chain-anchor is preferred for maximal transfection in vitro (49). It is not known at present whether the requirement of a cholesterol-anchor for cationic lipids to achieve high in vivo activity only applies to intranasal (or intralung) administration.

2. Double-chain hydrocarbon-anchored lipids. The majority of cationic lipids synthesized so far belong to this category which includes DOTMA, the first cationic lipid shown to efficiently transfect cells. After the introduction of DOTMA, Felgner et al. studied the effect of various chemical structure changes on the DOTMA molecule (Figure 2) (50). Substitution of methyl in the charged group with hydroxyalkyl was shown to improve the activity of the lipid (50). It was believed that the hydroxyl group facilitates a hydrogen bonding with DNA,



	R
DOTMA	CH_3
DORIE	$HO(CH_2)_2$
DORIE-HP	$HO(CH_2)_3$
DORIE-HB	$HO(CH_2)_4$
DORIE-HPe	$HO(CH_2)_5$



	R
DORI	$CO(CH_2)_7CH{=}CH(CH_2)_7CH_3$
DORIE	$(CH_2)_8CH{=}CH(CH_2)_7CH_3$
DMRIE	$(CH_2)_{13}CH_3$
DPRIE	$(CH_2)_{15}CH_3$
DSRIE	$(CH_2)_{17}CH_3$

Figure 2 Double-chain cationic lipids synthesized by Felgner et al. (From Ref. 50.)

resulting in efficient compaction of DNA. Introduction of the hydroxyl group was also thought to enhance membrane hydration. Upon varying the chain length of hydroxyalkyl moiety on the quaternary amine while keeping the remaining structure unchanged, it was shown that the activity of lipids decreased with increase in the hydroxyalkyl chain length (50). For example, lipid DORIE, which has an hydroxyethyl chain, was more active than lipid DORIE-HP, which has a hydroxypropyl chain. It was speculated that an increase in the number of carbon atoms in the hydroxyalkyl chain could increase the flexibility of the terminal hydroxyl group. This might lead to an inefficient interaction between the hydroxyl group and DNA, which, in turn, can cause reduction in transfection efficiency. The alkyl chain length can also influence the activity of the lipid. The order of efficiency was C14 : 0 (DMRIE) > C18 : 1 (DORIE) > C16 : 0 (DPRIE) > C18 : 0 (DSRIE) (50). Finally, they found that the activity of lipids can be affected by the nature of the linker bond. The lipid DORIE, which contains an ether linkage, was more active than lipid DORI, which contains an ester

bond. The lower activity of lipid DORI might be due to a faster degradation of lipid. This study established DMRIE as the most active lipid in this study (50). In mixtures with DOTMA-like lipids, DOPE was found to be the most effective helper lipid. Multilamellar vesicles were more efficient than small unilamellar vesicles in transfecting cells in vitro (50). Recently, a more efficient lipid, GAP-DLRIE, was reported by the same group (51). Compared with DMRIE, GAP-DLRIE has shorter alkyl chains and a primary amine instead of a hydroxyl group appended to the quaternary ammonium in the polar head portion. It is believed that the improved hydrophilicity and possible charge of the amino group provide the enhanced transfection efficiency (51).

In addition to the importance of the structure of the lipid itself, the lipid counterions also play an important role in determining the lipid activity. A recent study by Bennet and colleagues suggests that ions with highly delocalized anionic charge enhance transfection (52). The order of efficiency is bisulfate > trifluoromethylsulfonate > iodide > bromide > chloride > acetate, sulfate (52).

It should be noted that while structure-activity relationships can be deduced from some cationic lipids, the principles guiding the synthesis of a "magic lipid" may change when the delivery system changes. Several studies suggest that there is little correlation between in vitro and in vivo systems with regards to the structural requirement for cationic lipids (49,53). Different administration routes might also require different structural features of cationic lipids. Currently, the search for the "magic lipid" is still largely on a trial and error basis. Understanding of these differences will help in designing and synthesizing of more efficient cationic lipids.

B. Structure of Liposome/DNA Complexes

It is generally known that cationic liposomes interact with DNA via charge interaction. The interaction between the two components can be monitored using two fluorescence probes as in the so called lipid-mixing studies. Several studies suggest that DNA/lipid interaction is extremely fast, occurring within one minute after mixing (18). However, the maturation of lipid/DNA complexes may be a slow process. The structure of liposome/DNA complexes continues to change with time. In addition, the structure of liposome/DNA complexes is also determined by many other factors including lipid structure, charge ratio between lipid and DNA, and the respective concentrations of lipid and DNA. Depending on the pKa of the cationic lipid and the helper lipid, the pH can also affect the interaction of cationic lipid with DNA and the structure of the complexes. Several morphological studies using electron microscopy (EM) suggest that structural rearrangement of the lipids occurs when forming complexes with DNA and that liposome/DNA complexes are heterogeneous in structure (18–20). Using negative stain and metal-shadow EM, Gershon et al. found that at a DNA/

lipid ratio of 1 : 1, approximately half of the DNA molecules were bound to liposomes and the complexes were spherical (18). When the liposome concentration was increased, all the DNA became covered by liposomes and a smooth rod-like structure was revealed. Similar structures were later found by Sternberg et al. when they examined DC-chol : DOPE/DNA complexs by using freeze-fracture EM (19). This gave rise to the description of the so-called "spaghetti" and "meatball" structures (Figure 3). The tubular spaghetti-like structure and meatball-like spherical aggregates were also observed for DOTMA liposome/DNA complexes. They speculated that these structures were formed by a DNA-mediated fusion process and that "spaghetti" is composed of the DNA covered by a single lipid bilayer. This model was supported by two recent studies on lipid/DNA complexes using x-ray diffraction (XRD) and small-angle x-ray scattering (SAXS) (54,55). Both studies revealed a multilamellar structure with alternating lipid bilayer and DNA monolayers. A long periodicity of 6.5 nm was observed in the latter study (55), which is consistent with a lipid bilayer thickness of 4 nm and hydrated DNA helix of 2.5 nm. It should be noted that all of

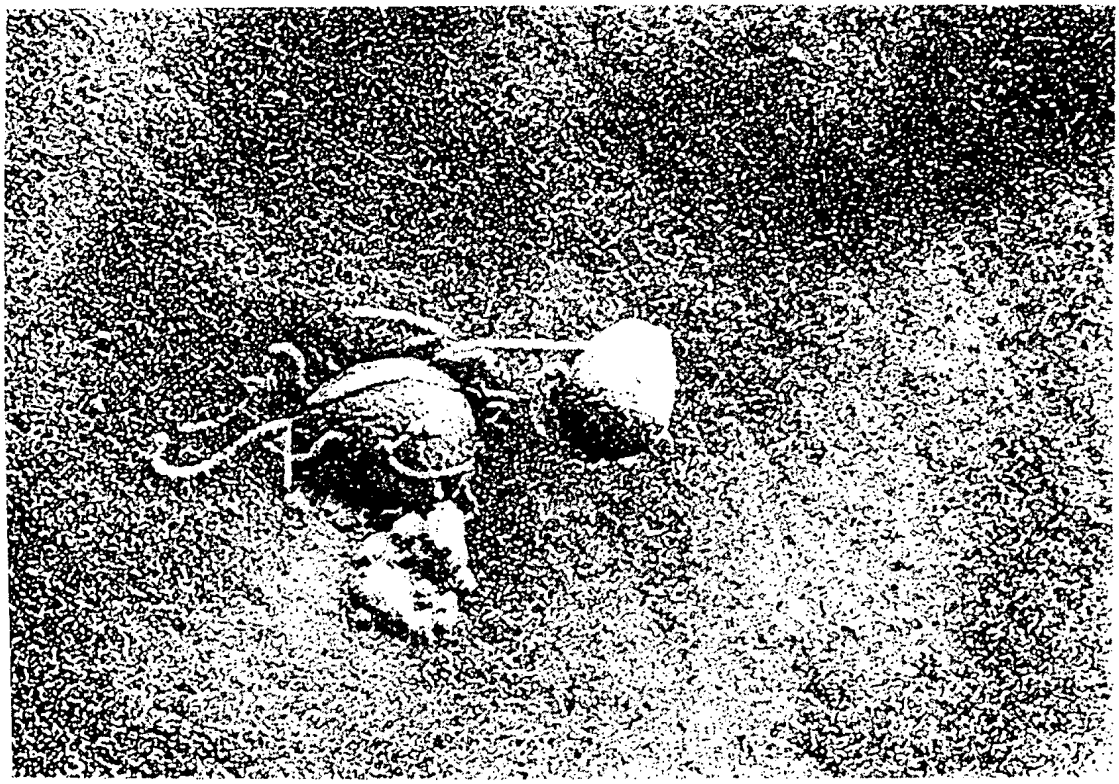


Figure 3 Freeze-fracture electron micrograph of DC-chol liposome/DNA complex. Shown here are the structures of "spaghetti and meatball." (From Ref. 19 with permission.)

the above studies have employed monovalent cationic lipids. DNA complexed with a mutivalent cationic lipid seems to adopt an entirely different structure. Rather than forming tubular structure and/or fused spherical aggregates, DNA is condensed into small, compact, non-aggregating particles (56; Sorgi et al., unpublished data). This might be due to the greater DNA-condensing capacity of the latter.

C. In Vivo Use of Cationic Liposome/DNA Complexes

Cationic liposome/DNA complexes have been delivered into living animals by different administration routes. Development of suitable vectors for each administration route requires the understanding of biological environment where interactions between liposome/DNA complexes with target cells take place. The following are a summary of all of the administration routes employed so far for in vivo gene delivery. Each will be discussed in the context of formulation consideration, efficacy, toxicity and potential applications.

1. Intratumor Injection

Intratumor injection is a widely used approach for direct gene transfer. While under some circumstance, naked plasmid DNA works better than what is delivered by a vector, cationic lipids are found in many studies to improve gene expression efficiency (57,58). However, the amount of lipids required to achieve maximal gene expression is far less than the optimal dose of lipids for in vitro transfection. At the optimal lipid/DNA ratio, the liposome/DNA complexes carry a net negative charge. This might be due to a different transfection mechanism when naked DNA or liposome/DNA complexes are administered locally. Plasmid DNA might be taken up by cells through a receptor-mediated endocytosis (59). Enhancement of gene expression by cationic lipids might be due to improved protection of DNA against enzymatic digestion. Partial neutralization of DNA by cationic liposomes might also decrease the electrostatic repulsion between plasmid DNA and its target cells. Finally, the liposome/DNA complexes may act as a slow release system, allowing released DNA to be taken up by cells over a prolonged period of time. Use of excess amounts of lipids might lead to an aggregation of complexes thus making cellular uptake of DNA and release of DNA from the complexes more difficult.

Toxicity studies demonstrate that intratumor injection is safe and does not cause any abnormalities in major organs (9). DNA is primarily localized in the injected tumor and occasionally detected in heart, kidney, lung and spleen. In a therapeutic study, a mammalian DNA expression vector coding for an H-2K^s mouse histocompatibility complex (MHC) class I protein was used with DC-chol liposome as a delivery vehicle (60). The injected mice had a different

haplotype of the class I MHC molecule (H-2K^d). A cellular immune response to the recombinant H-2K^s protein was evident in the animals injected with the *H-2K^s*-liposome complexes. The immune response attenuated tumor growth and caused complete tumor regression in some animals. Furthermore, introduction of *H-2K^s* into the primary tumor also induced a preventive effect against a second challenge of parent tumor cells at a distant site (60). Recently, direct gene transfer has also been employed to inhibit tumor growth by down-regulating the expression of oncogenes. For example, direct injection of DC-chol liposomes complexed with a plasmid expressing an antisense RNA against the *E6* and *E7* genes of papilloma virus resulted in a significant inhibition of the growth of HPV 16-positive C3 sarcoma in an animal model. Down-regulation of oncogenes has also been shown to inhibit the growth of other types of cancer such as head and neck carcinoma (He et al., unpublished data). Eventually, a strategy combining both immunological and nonimmunological approaches may be required to further improve therapeutic efficiency. Interesting is the discovery that in vivo lipofection of some tumors such as human ovarian cancer can be sensitized by pretreating the host with cis-platin, an anticancer drug (57). This observation may have a profound clinical implication as it suggests a sequential, combinational gene therapy protocol with cisplatin for human ovarian cancer.

2. *Intravenous Administration*

Intravenous gene delivery via cationic liposomes was first reported by Zhu et al. in 1993 (61). However, it is not until recently that researchers are able to obtain high and reproducible in vivo gene expression by iv injection (62–65). Several studies suggest that there is a significant difference between in vitro and in vivo (iv) transfection with respect to the optimal formulation. This will be addressed in more detail in Section IX of this chapter. Here, we only summarize the results of in vivo gene expression in several recent studies. All of the studies show gene expression in all major organs including heart, lung, liver, spleen, and kidney. Lung is always the organ with the highest level of gene expression. The level of gene expression ranges from pg to ng when the activity is expressed as the amount of gene product per mg of extracted tissue protein. Endothelial cells are the major cell type transfected. Gene expression lasts for about 1 week. For repeated injections, there is a unresponsive or poorly responsive period of 2 weeks between 2 injections during which high level of gene expression can not be achieved upon a second injection. It is likely that in vivo gene expression via iv administration of cationic liposome/DNA complexes is due to a first passage effect. However, it is still not clear how the complexes interact with blood components and transfect the endothelial cells.

3. *Intraperitoneal Administration*

Intraperitoneal administration of the liposome/DNA complexes can be potentially employed to treat diseases which affect the peritoneum. One of the appli-

cations is in the treatment of malignant metastasis such as tumor ascites. Several studies demonstrated that tumor ascitic cells could be efficiently transfected by ip administration of the DC-chol liposome/DNA complexes (66, Yang et al., unpublished data). Even solid tumors in the peritoneal cavity could be transfected (66). In the study by Yu et al., a nude mouse ascitic model induced by human ovarian cancer cells SKOV-3 was used (66). The optimal ratio between DNA and lipids was found to be 15 μg DNA/200 nmol lipids. The complexes thus formulated carried a net positive charge which might be required to neutralize the hostile factors in the ascites. Following optimization of the transfection protocol, a therapeutic experiment was then pursued (66). *E1A* was used as a therapeutic gene because introduction of *E1A* to SKOV-3 cells has been shown to bring about down-regulation of HER-2/*neu* and cellular growth inhibition in vitro. It was found that liposome-mediated *E1A* gene transfer significantly inhibited growth and dissemination of ovarian cancer cells in the treated mice; about 70% of these mice survived more than a year whereas all the control mice died within 160 days (66). In a subsequent safety study, mice received a single or multiple injections (5 consecutive days) of DC-chol liposome/*E1A* complexes. The highest dose for a single injection was 150 μg of DNA per mouse while the highest cumulative dose for repeated injections was 600 μg of DNA per mouse. Pathological studies revealed no major abnormalities in several major organs including brain, heart, liver, kidney, lung and spleen. No significant changes were found in the serum enzymes or other biochemical parameters (67). These studies have establish the basis of a clinical trial using DC-chol liposome/*E1A* complexes for treating human ovarian cancer.

4. *Airway Administration*

Airway administration of liposome/DNA complexes is potentially useful for the treatment of many pulmonary diseases, although current efforts are mainly focused on cystic fibrosis, an autosomal recessive disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The methods for delivery include intranasal administration, intratracheal instillation, and aerosol delivery. They differ in the amount of DNA which can be delivered to the airway or a specific segment. For example, in the study by Lee et al. using mice as a model, intranasal administration was shown to deliver about two-thirds of the material which could be achieved by direct intratracheal instillation (49). For aerosol delivery, liposome/DNA complexes can be delivered preferentially to nasal epithelium, upper airway, or distal airway by manipulation of the spray device. Normally, liposome/DNA complexes are formulated with an excess of positive charge which might be required to neutralize the hostile factors in the pulmonary surfactant. DOPE is required for most cationic lipids to achieve maximal delivery efficiency in vivo. Over the last few years, substantial efforts have been spent towards a search for more efficient lipids for

gene delivery to airway. Several newly synthesized cationic lipids are more efficient than the existing lipids for in vivo gene transfer (49,51). However, there is some inconsistency among different groups regarding the delivery efficiency of a given lipid. This reflects the complexity of an in vivo study and stresses the necessity of more carefully controlled experiments.

Both trachea and lung can be transfected by liposome/DNA complexes while several studies seem to suggest that alveolar cells are easier to transfect (49) and that upper respiratory tract is more refractive to transfection than the lower respiratory tract (13). Gene expression in mice is transient lasting for about 7–10 days. Yet, sustained, high level of expression can be achieved by repeated injections. A study on efficacy by Alton et al. (11) has demonstrated that DC-chol liposome-mediated gene (*CFTR*-cDNA) delivery could correct the ion transport defect in cystic fibrosis mice. Full restoration of cAMP stimulated chloride response was noticed in some animals and *CFTR* cDNA expression could be detected. Overall, a range of correction was seen with restoration of about 50% of the deficiency between wild-type and untreated cf/cf controls (11). Correction of the ion transport defect was also shown later in several other studies using different cationic liposomes (13,49).

Most studies showed little or no inflammation in the airway after liposome-mediated gene delivery. A recent study by the Genezyme group, however, demonstrated that intranasal instillation of lipid #67/DNA complexes into BALB/c mouse lungs induced a dose-dependent pulmonary inflammation which was characterized by infiltrates of neutrophils, and, to a lesser extent, macrophages and lymphocytes (68). The lesions in the lung were multifocal and were manifested primarily at the junction of the terminal bronchioles and alveolar ducts. The inflammation abated with time and there was no permanent fibrotic lesions (68). Specific immune response and activation of the complement were not found. However, it is not clear whether immune response could be developed by repeated administration of cationic liposome/DNA complexes.

5. *Other Administration Routes*

Several other administration routes have also been employed for in vivo gene transfer including intracranial injection, intramuscular administration, intradermal injection, and intravascular perfusion, among which intracranial administration is extensively explored. In the study by Schwartz et al. using lipospermine as a delivery vehicle, a low charge ratio between lipids and DNA was found to be optimal for intracranial gene delivery (69). Recently, in collaboration with Dr. During at Yale University, a novel LPD formulation composed of DC-chol/DOPE liposomes, polylysine and a LacZ reporter gene was prepared and injected into the brain of a rat. A high level of gene expression was found along the track of injection. Even cells several mm away from the site of injection

were transfected. Transfection efficiency of LPDI was much higher than that of LipofectAMINE (During et al., unpublished data).

6. *Clinical Trials*

More than a dozen clinical trials have been ongoing using cationic liposomes as a delivery vehicle. These trials are largely testing of safety and confirmation of in vivo gene expression. Three independent studies have shown gene expression when CFTR gene was delivered via cationic liposomes to nose of the patients with cystic fibrosis (12–14). Transient and partial correction of the nasal transepithelial ion transport defect has been found. To date, no report of gene transfer to the lung has been described. It is expected that transfection of lung will be more difficult owing to the presence of large amounts of viscous mucus in the airway of CF patients. Apparently, current formulations have to be improved before CF patients can benefit from the cationic liposome-mediated gene therapy. In contrast to cystic fibrosis trials, more encouraging results were found in the clinical trials of gene therapy for cancer patients. In a clinical trial of introducing a human *HLA B7* gene into human melanoma via DC-chol liposomes, complete regression of the injected tumor was observed in one out of five patients (15). In addition, metastatic lesions at distant sites displayed complete regression over the same time period (15). It was believed that expression of an allogeneic MHC antigen in the tumor elicited a tumor-specific as well as a nonspecific immunological response leading to tumor regression. In another clinical trial using a non-immunological approach, tumor regression was also found in ovarian cancer patients receiving ip injection of DC-chol liposomes complexed with *E1A* gene (Hung et al., unpublished data). These results look encouraging, however, more preclinical and clinical studies are required before any conclusion regarding the efficacy of gene therapy can be drawn.

D. Mechanism of Transfection

Cationic liposomes are also used as a tool to study the mechanism of transfection. However, conclusions obtained from cationic lipsomes also apply to other lipidic vectors such as neutral and anionic liposomes, although some differences do exist. Interaction of liposome/DNA complexes with cells can be divided into three steps: entry of DNA into cells, escape of the DNA into cytoplasm and entry of plasmid DNA into the nucleus.

1. *Entry of DNA into Cells*

Entry of DNA into cells is the first step for transfection. Neutral and anionic liposomes bind weakly to cells through an inefficient, nonspecific interaction. However, a targeting ligand can be introduced to improve their interaction with

cells. Cationic liposomes are generally believed to interact with cells through a nonspecific charge interaction. A study by Zabner et al. demonstrated that the process of DNA entry into cells was relatively slow (70). There was a correlation between the percentage of cells taking up DNA and the percentage of cells expressing transgene. Cell lines taking up more DNA gave higher level of gene expression than did the cells which took up less DNA. This result suggests that in some cells the uptake of liposome/DNA complexes may be an important barrier to transfection. The factors governing the binding of the liposome/DNA complexes to cells and their subsequent cellular uptake are not clearly understood. A recent study suggests that the amount of negative charge on cell surface plays an important role in determining the interaction between cationic liposome/DNA complexes and cells and therefore influences the efficiency of gene expression. Cells with more negative charge on the surface are easier to transfect (71). It is interesting to note that tumor cells tend to be more negatively charged than normal cells of the same type. This might explain why tumor cells are relatively more transfectable. However, it should be noted that other factors besides cellular uptake of DNA may also play important roles in transfection. A study in our laboratory showed that a SSC25 cell line and its subline, SSC25-CP, took up equal amount of DNA if delivered by DC-chol liposomes. Yet, these two cell lines differed greatly in transgene expression (Chang and Huang, unpublished data).

2. Escape of DNA into the Cytoplasm

Previous studies have suggested that endocytosis is the major mechanism for pH-sensitive liposome-mediated intracellular delivery of MHC class I-restricted antigens, dipheria toxin fragment A (DTA), and plasmid DNA (72). For cationic liposome-mediated gene delivery, fusion of cationic liposomes with the cell membrane was initially proposed as the major pathway of internalization of liposome/DNA complexes (73). Later studies with EM and other biological assays suggest that cationic liposome/DNA complexes are taken up by cells mainly via an endocytosis mechanism (74–77). Zhou and Huang have studied the intracellular trafficking of DNA complexed with cationic liposomes composed of DOPE and lipopolylysine (LPLL) (76). LPLL liposomes condense DNA to form electron dense particles which can be positively identified by negative stain or thin section EM. A majority of the dense particles were found in the vesicular compartments. Disruption of endosomal compartment was also visualized, the frequency of which was much higher than that of direct penetration thorough the plasma membrane (15% vs 0.7%). When cells were treated with chloroquine, about 57% of the observed endosomes displayed destabilized morphology, a 42% increase compared with cells without treatment. This was in accordance with the result of a bioassay which showed a 6-fold increase in

transfection efficiency after chloroquine treatment. Interestingly, if dioleoylphosphatidylcholine (DOPC) instead of DOPE was used as a helper lipid, less than 1% of complex-containing endosomes were destablized (76). Membrane enriched with DOPE has a strong tendency to form an inverted hexagonal phase, a structure frequently seen in regions where membrane fusion takes place (78). Thus, it is believed that DOPE facilitates the disruption of endosomes and the release of free DNA or liposome/DNA complexes into the cytoplasm. Recently, it has been speculated that charged lipids may segregate away from neutral lipids when mixing cationic liposomes with plasmid DNA. Likewise, it was speculated that some lipids such as DC-chol may dissociate from the vector when they become fully charged, such as in the acidic endosomal enviroment. The presence of DOPE may facilitate the process by providing an instability in the bilayer. Released cationic lipids may then participate in the process of endosome disruption via their detergent properties. Currently, several approaches have been attempted to intervene with the intracellular trafficking of liposome/DNA complexes, in an effort to improve their transfection efficiency. One of the approaches is the use of a fusogenic peptide (79). Fusogenic peptides are usually anionic, water soluble, and random coil in conformation at physiological pH, but undergo a transition to an amphipathic alpha-helix when the pH is reduced. It was shown that at low pH values the peptides became associated with lipid bilayer and caused pH-dependent fusion of small liposomes (72). It is expected that such peptides facilitate the process of endosome disruption when co-delivered with the liposome/DNA complexes, resulting in an improved cytoplasmic delivery of DNA. We found that gene delivery efficiency by several cationic liposomes can be enhanced by 6–14 fold when co-delivered with the fusogenic peptide, GLFEALLELLESLWELLLEA (Li and Huang, unpublished data).

3. Entry of DNA into the Nucleus

Most of the expression systems rely on the host endogenous transcriptional machinery, i.e., the RNA polymerase II or III and the associated regulatory factors for gene expression. Therefore, entry of DNA into the nucleus is essential following its release into cytoplasm. Unfortunately, little is known about the mechanism by which DNA moves from cytosol into the nucleus, except that this process is very inefficient (80). Attempts to conjugate a nuclear localization signal peptide to DNA only led to limited improvement in transfection (81). Recently, several groups have tried to develop plasmid DNA with improved expression activity to compensate for its inefficient nuclear transport (82,83). For example, the Vical group reported a 1000 fold increase in gene expression by modifying the uncoding sequences of a plasmid DNA (82). A similar study was later reported by the Genezyme group (83). Another alternative is the use

of a cytoplasmic expression system, i.e., T7-based expression system. It is composed of a bacteriophage T7 promoter and its corresponding RNA polymerase and is independent of the host endogenous transcriptional machinery. T7 RNA polymerase remains primarily in the cytoplasm owing to the lack of a nuclear localization signal; therefore, nuclear transport of the plasmid DNA is not necessary. Rapid and transient expression of a CAT reporter gene, pT7-CAT, was found when co-delivered with purified T7 RNA polymerase (84). When pT7-CAT was co-delivered with a T7 RNA polymerase regeneration system (an autogene such as pT7 AUTO2C$^-$ or pCMV/T7-T7pol.), strong and sustained CAT expression was achieved, which was greater than that seen with the nuclear expression system such as pUCSV2CAT or pCMVCAT (85,86). Eventually, a gene transfer protocol employing both expression systems could be utilized to further improve transfection efficiency. This possibility is being examined in our laboratory.

4. Uncoating of DNA

It is hypothesized that DNA must dissociate from the polycation before transcription could take place. However, it was not until recently that DNA uncoating was brought to attention. Using microinjection, Zabner et al. clearly demonstrated that DNA is transcriptionally inactive when complexed with cationic liposomes. Cationic liposomes inhibit gene expression in a dose-dependent manner (70). The exact mechanism by which DNA is released from the liposome/DNA complexes is not clear. It was thought previously that release of DNA takes place in the nucleus due to displacement of the plasmid DNA from cationic lipids by genomic DNA (87). However, a recent in vitro study showed that neither polycation such as spermidine and histone, nor nucleic acids can bring about dissociation of preformed liposome/DNA complexes. Instead, plasmid DNA can be readily released from liposome/DNA complexes by anionic liposomes containing compositions that mimic the cytoplasmic-facing monolayer of the plasma membrane (88). It is speculated that DNA might be similarly released by anionic lipids inside the cells. Destabilization of endosomal membrane, which is initiated by liposome/DNA complexes after their endocytosis, may bring about a flip-flop of anionic lipids predominately located on the cytoplasmic face of the membrane. The anionic lipids may laterally diffuse into the complexes and form charge-neutralized ion pairs with the cationic lipids, resulting in displacement of the plasmid DNA from liposome/DNA complex (88). Our study with the T7 cytoplasmic expression system also indicated the importance of DNA uncoating but only when DNA is condensed by a polycation. Simply coating plasmid DNA with a monovalent cationic lipid did not inhibit its accessibility to T7 RNA polymerase (89). This result suggests a difference between the two different expression systems in their requirement for a delivery vehicle.

The mechanism regarding in vivo transfection is also poorly understood. With different administration routes, different mechanisms might be involved. Most likely, the liposome/DNA complexes will change in their physical properties after in vivo administration and the extent of change may be related to their interaction with biological fluids. When there is limited contact with biological fluids such as with an intratumor injection, the liposome/DNA complexes may not change appreciably in its physical properties before they encounters the tumor cells. In contrast, when liposome/DNA complexes are administrated into the blood, the size, and structure, as well as the net charge of liposome/DNA complexes, are expected to change significantly before they can reach the target cells. Several studies indicate a significant discrepancy between in vitro and in vivo systems and suggest that an in vivo study should be performed independently in developing vectors for in vivo gene transfer. We believe that in vitro study is still informative if it is conducted in the context of the in vivo situation. For example, transfection efficiency of a lipid can be tested in the presence of serum. Active components can also be purified after the exposure of liposome/DNA complexes to serum and then examined for their interactions with cells. These studies might add greatly to our understanding of how cells are transfected in vivo.

E. Existing Problems with Cationic Liposomes

While early laboratory studies and clinical trials have demonstrated the potential of cationic liposomes in gene therapy, they have also indicated the inadequate efficiency of the first generation cationic liposomes. Typically, cationic liposomes are several orders of magnitude lower than viral vectors in transfection efficiency. Low efficiency of cationic liposomes is probably related to each step of the transfection process, one of which may be inefficient cellular uptake. Cationic liposomes, especially those composed of monovalent cationic lipids, cannot condense DNA efficiently. As mentioned previously, complexation of cationic liposomes with DNA usually results in the formation of a spaghetti-structure together with a spherical structure with a tendency to aggregate. Whether any of these structures is responsible for transfection is not clear at present. Yet, considering the size of the aggregated and fused complexes, uptake of these particles through the mechanism of endocytosis is expected to be inefficient. Compared with virus, plasmid DNA complexed with cationic liposomes is also less efficiently protected from enzymatic digestion. These, together with other factors, might explain the low efficiency of some cationic liposomes.

Serum-sensitivity is another drawback associated with cationic liposomes. It is generally known that lipofection relies on a slight excess of net positive charge of lipid/DNA complexes to efficiently interact with negatively charged cell membrane. Interaction between liposome/DNA complexes and anionic mol-

ecules in the serum would neutralize the positive charges and decrease the performance of liposome/DNA complexes. This was confirmed in an in vitro study with DC-chol liposomes. Addition of serum into DC-chol liposome/DNA complexes greatly decreased the amount of cellular uptake of DNA and transfection efficiency (90). Anionic molecules in the serum might also affect the integrity of cationic liposome/DNA complex. A recent study showed that like anionic liposomes, water soluble molecules with a high negative charge density such as dextran sulfate and heparin can release DNA from liposome/DNA complexes (88). It is speculated that DNA might be released from liposome/DNA complexes in the blood by a similar mechanism, rendering DNA more susceptible for enzymatic digestion. These problems, together with other problems such as rapid clearance by reticuloendothelial system (RES), severely limit systemic application of cationic liposomes for in vivo gene transfer.

Current efforts to overcome these limitations have been largely based on our understanding of the structure of viruses and the mechanism by which viruses escape various barriers in productive infection. In the following section, we will describe several novel formulations which can potentially solve one or several of the above problems.

V. CATIONIC LIPOSOME-ENTRAPPED, POLYCATION-CONDENSED DNA (LPDI)

Polycations such as poly-L-lysine are known to efficiently condense DNA. The morphology of the polycation/DNA complex has been extensively studied and the features of this complex are well characterized. A polycation/DNA complex often appears as a toroid and the size of the particle is about 50 nm (91). The binding of DNA to a polycation is stoichiometric and the condensation process is highly cooperative (92). This is in contrast to the formation of a spaghetti structure accompanied by large aggregates when DC-chol liposomes are complexed with DNA. Considering the mechanism of lipofection and the difference in the ability to condense DNA between monovalent cationic lipids and polyamines or multivalent cationic lipids, Gao and Huang hypothesized that introduction of cationic polymers at appropriate ratios to the mixture of the DC-chol/DOPE cationic liposomes and DNA might alter the overall structure of the liposome/DNA complexes, and thus change the biological activity of the complexes. This idea was tested by examining the tranfection efficiency of a reporter gene complexed with cationic liposomes alone, or cationic liposomes plus various amounts of polymer. Various lipsomes including Lipofectin, LipofectAMINE, DC-chol liposomes, and several different polymers were evaluated. It was found that the use of liposomes together with a cationic polymer consistently gave higher transfection activity than did the corresponding liposome/

DNA complexes. The potentiation effect (2–28 fold) was observed in a number of cell lines in vitro. In particular, cells difficult to transfect with liposome/DNA complexes could be transfected when a cationic polymer was included (22).

Analysis of the mixture on a sucrose gradient ultracentrifugation showed the existence of several populations which differ in the amount of lipids associated with the complexes. Those fractions enriched with lipids were more potent than those with less amounts of lipids. However, the activity of the fraction lacking sufficient amounts of lipids could be significantly improved when free DC-chol/DOPE liposomes were added. The purified complexes were several fold more efficient than the unpurified mixture in transfecting cells in vitro. In addition, the purified complexes were less toxic.

The structures of purified complexes were studied by negative-stain EM. Various shapes of electron dense structures ranging from elongated rod-shaped to ball-shaped particles were found. This was related to the amount of lipids associated with the particles. Typically, the complexes giving the highest level of activity appeared as compact spherical particles with a mean diameter of < 100 nm (Figure 4). The core of the particles was heavily stained and might represent polylysine-condensed DNA. Some of the ring structures had typical characteristics of a membrane staining pattern. The purified complexes have been named LPDI particles (22). A possible explanation for the formation of such particles is that the highly efficient interaction between DNA and PLL results in the formation of a partially condensed particle. Association of liposomes with the condensed particles then leads to lipid structural rearrangement and the formation of a lipidic shell on the surface of the particles.

There are several possibilities which may explain the higher performance of LPDI particles compared with the corresponding liposome/DNA complexes. First, unlike the DC-chol liposome/DNA complexes which are large and heterogeneous in size, LPDI particles are highly compact with a size of less than or close to 100 nm. These particles would be more favorable for entering cells via an endocytosis pathway, which is the major mechanism responsible for the cellular uptake of liposome/DNA complexes. Therefore, this formulation is particularly suitable for transfecting those cells which lack other DNA uptake mechanisms. This might explain why some cells difficult to transfect with liposome/DNA complexes could be efficiently transfected by LPDI particles. Second, LPDI particles offer better protection of DNA from enzymatic digestion. DC-chol liposomes at suboptimal ratio did not protect the DNA from DNAse in the serum. Even at the optimal ratio, DNA was not completely protected. In contrast, LPDI offered complete protection of the supercoiled conformation of plasmid DNA. Therefore, greater amounts of active DNA could be delivered to cells by LPDI. Finally, PLL may mimic the nuclear localization signal and facilitate the nuclear transport of DNA. All these favorable characteristics make LPDI a novel, highly efficient non-viral gene delivery vehicle. The drawback of DC-

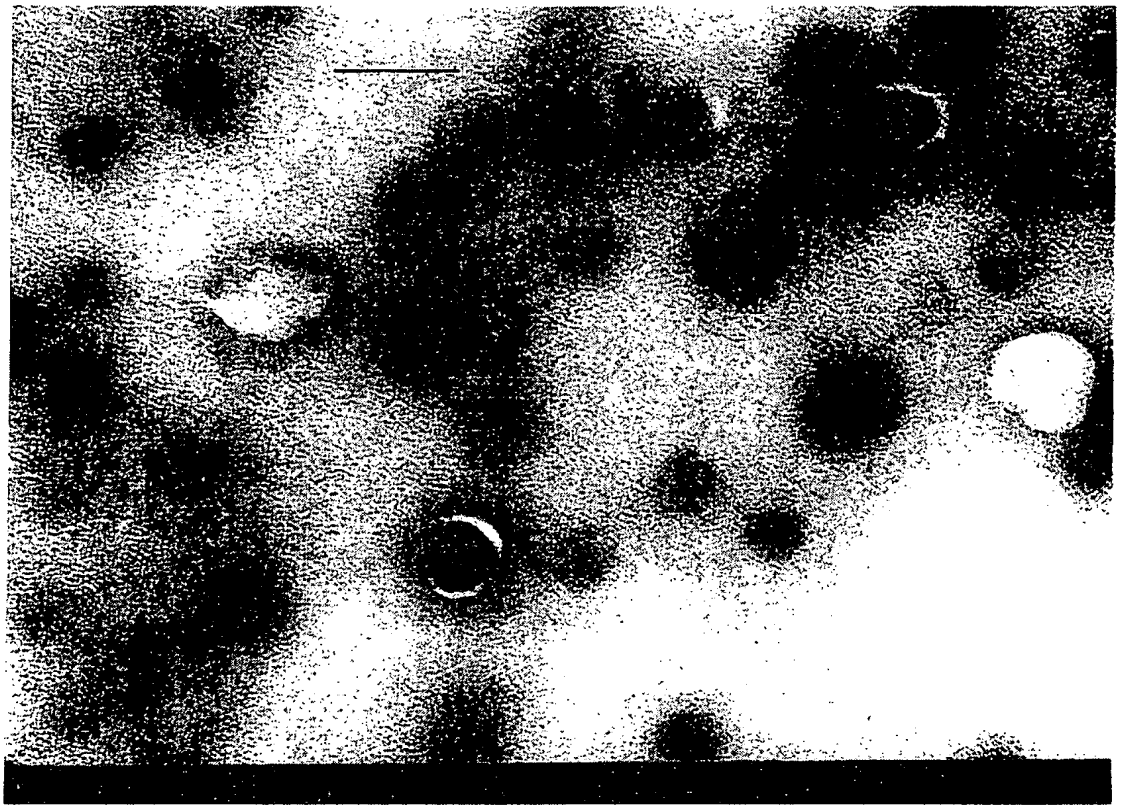


Figure 4 Electron micrograph of LPDI particles. The complex was prepared from 20 μg DNA, 10 μg PLL (MW 26,500) and 800 nmol DC-chol/DOPE liposomes (2/3, m/m), purified with sucrose gradient ultracentrifugation, and examined with negative staining EM. Bar = 100 nm. (From Ref. 22 with permission.)

chol-containing LPDI particles is their sensitivity to serum due to the net positive charge of the particles. LPDI particles also work in a cell-type nonspecific manner and require purification for optimal activity.

VI. ANIONIC LIPOSOME-ENTRAPPED, POLYCATION-CONDENSED DNA (LPDII)

In an effort to seek an iv injectable, targetable vector for gene transfer, another new lipidic vector was also developed in this laboratory. Structurally, it is similar to LPDI. Both contain a highly condensed core composed of PLL and DNA and a lipid shell. Therefore, this novel vector is named LPDII (23). The protocol and the possible mechanism for the formation of LPDII is shown in Figure 5. DNA is first condensed with PLL with positive charge in moderate excess.

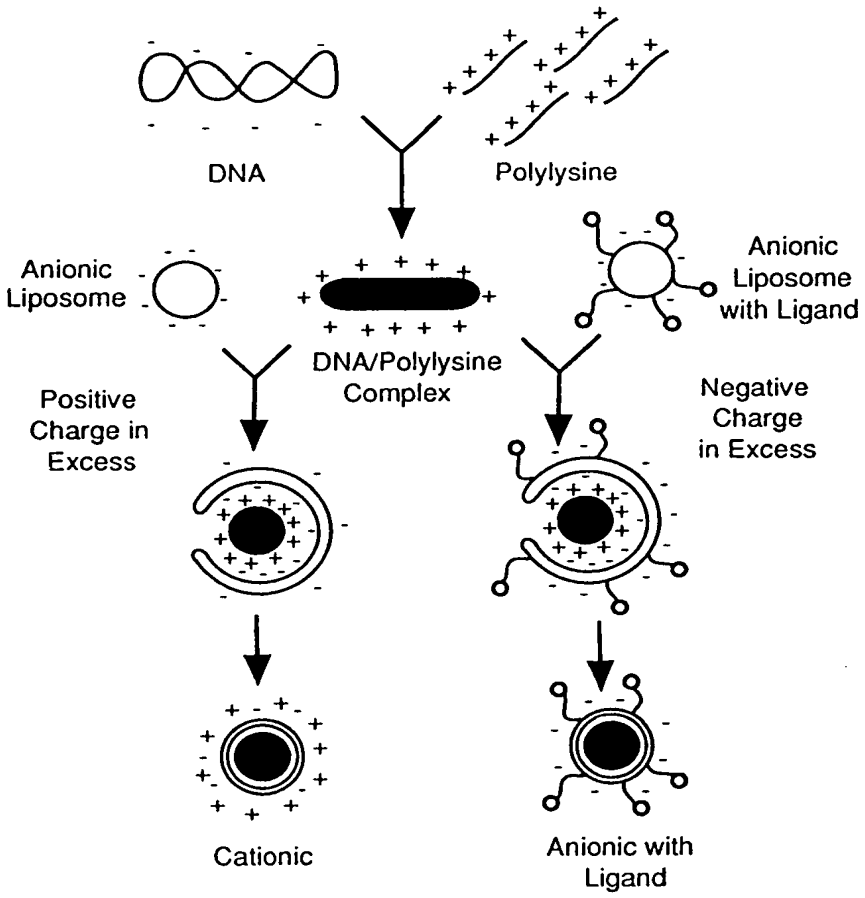


Figure 5 Possible mechanism for the formation of LPDII particles. The targeting ligand is folate. (From Ref. 23 with permission.)

The resulting cationic complex is then mixed with anionic liposomes carrying a targeting ligand. In the initial study, pH-sensitive liposomes composed of DOPE/CHEMS/folate-PEG-DOPE were used. Folate was chosen as a targeting ligand since many human tumors, especially ovarian carcinoma, overexpress folate-binding protein or folate receptor (93,94). Folate is a low molecular weight ligand with little or no immunogenecity, and has a high affinity and specificity for its receptor (95). Folate-mediated targeting of anticancer drugs have been well demonstrated in vitro as well as in animal models (96,97).

The net charge of LPDII particles is related to the lipid/DNA ratio. At low lipid/DNA ratios, the particles carried a net positive charge. Transfection and uptake of cationic LPDII were independent of folate receptor and did not require a pH-sensitive lipid composition. When sufficient amounts of lipids were used, the particles became slightly negatively charged. Transfection and uptake of

anionic LPDII were folate-receptor dependent, partially blockable by excess free folate, and required a pH-sensitive lipid composition (23).

Like LPDI, the formation of LPDII particles is likely due to fusion of liposomes and rearrangement of lipids after mixing cationic PLL/DNA complex with anionic liposomes. The presence of a targeting ligand did not seem to hinder the formation of LPDII particles. Recently, another LPDII was developed by mixing cationic PLL/DNA complex with anionic liposomes containing a high molecular weight ligand, transferrin. Transferrin-targeted LPDII was shown to selectively deliver a reporter gene to myoblasts and myotubes in vitro (98).

The advantages of LPDII are that, besides being highly compact, LPDII preparation does not require purification and is a single-vial formulation. Compared with traditional anionic and neutral liposomal vectors, DNA is highly condensed in LPDII and is quantitatively encapsulated without the requirement of using excess amounts of lipids. Finally, since anionic liposomes are relatively compatible with biological fluids, LPDII can be potentially used for tissue-specific gene delivery. The existing problem with current LPDII formulation is the serum-sensitivity, as incorporation of serum protein into LPDII can stabilize the bilayer resulting in the loss of pH-dependent fusogeneic activity. This problem might be eliminated by the use of a pH-sensitive but serum-resistant fusogenic peptide. This possibility is being examined in this laboratory. Another potential problem is the difficulty in preparing a concentrated sample. The current protocol for preparation of LPDII requires pre-condensation of DNA by PLL. Yet, formation of small condensed particles proves to be difficult at high concentration due to the strong interaction between polycation and DNA. Preparation of a concentrated sample might require a concentrating step after a diluted LPDII is formulated.

VII. CATIONIC MICELLES AND EMULSIONS

A recent study by Liu et al. showed that addition of an appropriate surfactant into the lipid components could prevent the formation of large aggregates of emulsion/DNA complexes (24,25). Three different types of nonionic surfactants, Tween 80, Span 80, and Brij 78, were examined. All could serve as good emulsifiers for emulsions composed of castor oil, DC-chol and DOPE. However, only Tween 80 could efficiently prevent the formation of large aggregates upon mixing of cationic emulsions with plasmid DNA. In contrast to DC-chol liposomes, in vitro transfection by emulsions was not highly dependent on the presence of DOPE. As shown in Table 1, inclusion of DOPE into the Tween 80-containing emulsion only gave a two-fold increase in transfection activity. Formulation #10, a cationic micelle, could also efficiently transfect cells in vitro. More importantly, in vitro transfection by Tween 80-containing emulsions and

Table 1 Effect of Lipid Composition on Transfection Activity

Formulation number	Composition (mg)					RLU/well $\times 10^{-7}$	Total proteins (μg/well)
	Oil	PC	DOPE	Tween 80	DC-chol		
1	0.25	0.25	—	0.125	0.75	15.9 ± 2.5	57 ± 1
2	—	0.25	—	0.125	0.75	3.2 ± 0.6	62 ± 1
3	0.25	—	—	0.125	0.75	17 ± 0.7	48 ± 2
4	0.25	0.25	—	—	0.75	2.4 ± 0.1	62 ± 3
5	0.25	0.25	—	0.125	—	0	61 ± 1
6	0.25	—	0.25	0.125	0.75	36.3 ± 3.2	55 ± 4
7	—	—	0.25	0.125	0.75	14.0 ± 0.5	61 ± 2
8	0.25	—	0.25	—	0.75	4.0 ± 0.5	59 ± 1
9[a]	—	—	0.6	—	0.6	10.7 ± 2.1	58 ± 3
10	—	—	—	0.25	0.75	25.9 ± 1.2	54 ± 4

Lipid components were mixed with the indicated amounts and formulations were prepared in 1 ml of PBS (pH 7.4). Transfections in BL-6 cells were performed using 2 μg of pCVM-Luc and 16 μl of each formulation, according to the procedure described in the method section. 10 μl of cell extracts from transfected cells were used in the assay for the luciferase activity. The level of enzyme activity in each well is presented as RLU for the total amount of extracted proteins.

[a]This is the DC-chol/DOPE liposome formulation. Taken with permission from Ref. 24.

micelles was not affected by serum. No significant difference in toxicity was found between DC-chol liposome/DNA complexes and DNA complexed with cationic emulsions or micelles.

The detailed mechanism by which Tween 80 stabilizes the lipid/DNA complexes is not clear. Tween 80 contains 3 oligo(ethylene oxide) groups in its polar head. The branched hydrophilic segments of Tween 80 may provide a steric hindrance by covering a relatively large area on the surface of the emulsions, preventing aggregation of emulsion/DNA complexes and their interaction with serum. Span 80 has the same hydrophobic part as Tween 80 but lacks the oligooxyethylene portion. Brij has only a single polyoxyethylene chain as compared to the branched oligooxyethylene chains in Tween 80. Therefore, Span 80 and Brij 78 may not provide sufficient steric protection, which might explain why these two detergents can not efficiently stabilize the emulsion/DNA complex. This hypothesis, however, is not consistent with the efficient interaction of Tween 80-containing emulsion/DNA complexes with cells. Another possibility is that the cationic emulsions undergo structural rearrangement upon mixing with plasmid DNA. Interaction of cationic lipids with DNA may result in charge neutralization and formation of a hydrophobic complex. Such a hydrophobic complex could be solubilized in the oil core of emulsions. More structural and physical studies are needed in order to better understand the interaction of cationic emulsions with plasmid DNA. So far it is not known whether targeted gene delivery via cationic emulsions is possible.

VIII. OTHER LIPIDIC SELF-ASSEMBLING SYSTEMS

Recently, it was shown that a hydrophobic lipid/DNA complex can be prepared in the absence of preformed liposomes (26–28). This hydrophobic complex can be isolated in an organic phase and used as an intermediate in the preparation of well-defined particles. A typical process for preparation of a hydrophobic lipid/DNA complex is as follows. Cationic lipids and plasmid DNA are solubilized in a Bligh and Dyer monophase consisting of chloroform/methanol/water (1 : 2.1 : 1). Subsequently, the sample is partitioned into an aqueous phase and an organic phase by further addition of chloroform and water. It is believed that binding of DNA to cationic lipids results in charge neutralization and extraction of the complex into the organic phase. Both monovalent and multivalent cationic lipids can form a hydrophobic complex with DNA and the amount of DNA which can be recovered from the organic phase is proportional to the amount of input lipids (28). Use of excess amounts of cationic lipids give a high yield of the lipid/DNA complex but introduces the problem of contamination of the complex with free cationic lipids. The lipid/DNA complex might serve as a unique intermediate in the preparation of lipidic gene delivery systems. For example,

the complex can be used in the preparation of oil/water emulsions. The complex can also be dissolved in alternative solvents for the preparation of membrane structures via a reverse-phase evaporation technique. There are several advantages of this system over liposome/DNA complexes for gene delivery. First, interaction of cationic lipids with DNA is a better defined process and the complex might be more homogeneous in structure. Second, formation of particles between the lipid/DNA complex and other lipids or among the complexes themselves is driven by hydrophobic interactions, and therefore, aggregation caused by electrostatic interactions is minimized. Finally, the surface properties of particles can be easily manipulated. Neutral or anionic particles could be prepared by incorporating appropriate lipids into the complex. A targeting ligand could also be used for targeted in vivo gene delivery. These favorable characteristics of this novel system are not shared by cationic liposome/DNA complex.

A. Reconstituted Chylomicron Remnants for In Vivo Gene Delivery

The reconstituted chylomicron remnants (RCR) recently developed in our laboratory are one demonstration of the use of a hydrophobic lipid/DNA complex for the preparation of well-defined particles (29). 3β-[N′,N′,N′-trimethylaminoethane)-cholesterol iodide (TC-chol), a quaternary ammonium derivative of DC-chol was employed to form a hydrophobic complex with DNA. DC-chol was not chosen because it contains a tertiary amino head group which is only partially ionized at neutral pH. The hydrophobic TC-chol/DNA complex extracted from the organic phase was then incorporated in RCR by emulsifying with appropriate amounts of triglyceride, l-α-phosphatidylcholine (PC), lysophosphatidylcholine (lyso PC), cholesterol (Chol) and cholesteryl oleate in a 70: 22.7: 2.3: 3.0: 2.0 weight ratio. After extrusion, the size of RCR was approximately 100 nm with a DNA incorporation efficiency of about 60% or greater. The RCR are stable and give a high level of gene expression upon injection from the portal vein. When a luciferase reporter gene was used, gene expression was found in all major organs including lung, heart, liver, spleen and kidney with the highest level of gene expression found in the liver. At a dose of 100 μg DNA per mouse, approximately 10 ng luciferase protein per mg extracted tissue protein could be detected in the liver. The level of gene expression by RCR was about 100 fold higher than that when naked plasmid DNA was used (29). Gene expression is transient and lasts for one week. However, long term expression can be achieved by repeated injections and/or by using a plasmid with extended lifetime in transfected cells (Hara and Huang, unpublished data). The existing problem with this formulation is the low efficiency when injected intravenously, only limited expression is found in the liver. This might be due to the lack of a targeting ligand. Addition of apo E or other ligands may improve the efficiency of iv delivery of gene to the liver by an efficient mechanism of receptor-medi-

ated cellular uptake. This possibility is currently being examined in this laboratory.

B. Stable Lipid/DNA Particles Prepared in Detergents

Bally et al. proposed that while a hydrophobic lipid/DNA complex can be used as an intermediate for the preparation of well-defined particles such as emulsions, the complex would adopt a unique macromolecular structure in the absence of stabilizing factors. Formation of the particles would be a process of self-assembling and be driven by hydrophobic interactions as opposed to charge-mediated interactions between cationic liposomes and DNA. This hypothesis was tested by forming a lipid/DNA complex in n-octyl β-D-glucopyranoside (OGP), a nonionic detergent. The detergent was then removed by dialysis (99). In their study, dioleoyldimethylammonium chloride (DODAC) was used as a cationic lipid. It was found that the concentration of OGP was critical in determining the behavior of the lipid/DNA complex produced. When the OGP concentration was close to the critical micellar concentration (CMC) of the detergent (20 mM), inter- and intra-molecular forces drove the spontaneous formation of a particulate structure. The size of the particles was about 100 nm and did not increase significantly after dialysis if the charge ratio (+/−) was 2 or greater (93). No aggregation was observed even after storage over 2 months. At a higher detergent concentration (100 mM), the lipid/DNA complex did not form particles until after dialysis. Continuous dialysis resulted in the formation of large, visible aggregates (99). When the two different types of particles were examined for their transfection efficiency, it was surprise to find that the small particles were not active while the aggregates showed an activity comparable to that of the corresponding liposome/DNA complexes. Poor activity of the small particles was thought to be due to their inefficient physical association with cells (99). In an independent study by a different group, a similar approach was employed to prepare the lipid/DNA complex (100). DOSPA was used as a cationic lipid and another neutral lipid, DOPE, was also used. Mixing of the lipids with DNA in the presence of 1% octylglucoside followed by dialysis resulted in the formation of homogeneous particles with an average diameter of 250 nm. These particles were more efficient than the corresponding liposome/DNA complexes in transfecting cells in vivo (101) as well as in vitro (95). The reason for the discrepancy between the two studies is not clear at present. It might be due to the difference in the cationic lipids used. Other factors such as the use of DOPE in the latter study might also contribute to the difference. More structural and functional studies are needed to clarify this question.

IX. OVERCOMING SERUM-SENSITIVITY OF LIPIDIC VECTORS

Serum can affect the performance of all lipidic vectors. For example, one common problem shared by all lipidic vectors after exposure to serum is their rapid

clearance from the blood. However, cationic lipidic vectors distinguish themselves from other lipidic vectors in at least two different ways. First, unlike neutral or anionic lipidic vectors, cationic lipidic vectors interact with cells mainly via charge interaction. Therefore, charge neutralization of cationic vectors by serum is one of the major mechanisms underlying their sensitivity to serum. Second, DNA might be released from cationic lipidic vectors by anionic molecules in the serum, a problem not shared by other lipidic vectors. Apparently, systemic application of cationic lipidic vectors is of more problem than other lipidic vectors. One solution to these problems is to change the cationic properties of the vectors such as with the development of RCR. This strategy is particularly useful when tissue-specific gene delivery is desired. Another way is to optimize the cationic vectors in such a way that they still retain their cationic properties but become serum insensitive. The latter possibility has been recently demonstrated in several different studies (24,25,62–65). For example, serum sensitivity can be overcome by using appropriate lipid compositions (cationic lipid and/or helper lipid) and/or by choosing an appropriate lipid/DNA ratio. Size of cationic liposomes was also found to be important in determining their sensitivity to serum. In the following, we will summarize all of the identified factors which affect serum-sensitivity of lipofection.

A. Concentration of Input Liposomes and DNA and Incubation Time of the Mixture

A typical protocol for in vitro transfection is to mix diluted DNA and diluted liposomes in serum-free medium. The mixture is then incubated for 10–30 min prior to being applied to cells. Addition of serum mostly inhibits the transfection efficiency. One study showed that serum-sensitivity of in vitro lipofection can be overcome by formulating the liposome/DNA complexes at a high concentration of liposomes and DNA (102). It was believed that the formation of serum-resistant complexes is a slow process which can be facilitated by increasing the concentration of lipid and DNA. This hypothesis was in agreement with our study which showed that serum-resistant complexes can be prepared using standard protocol but prolonging the incubation time (Yang and Huang, unpublished data). It appears that the formation of the serum-resistant liposome/DNA complexes is a process of maturation which can be achieved either within a short period of time by using a high concentration of liposomes and DNA or by longer incubation of diluted liposomes and DNA. Identification of the structure of the "matured" complexes might help in understanding the mechanism of in vitro transfection in the presence of serum.

B. Size of Cationic Liposomes

A previous study by Felgner et al. demonstrated that multilamellar vesicles (MLV) were several fold more efficient than small unilamellar vesicles (SUV)

in transfecting the cells in vitro (50). Recently, a more striking difference between SUV and MLV was found when a gene was delivered via cationic liposomes intravenously (62). MLV were about 10–100 fold more efficient than SUV in transfecting cells in vivo. In addition, SUV of larger sizes are more efficient than SUV of smaller size. So far, no structural explanation has been provided as to why MLV are more efficient than SUV for intravenous gene delivery. It was believed that, with MLV, DNA might be more efficiently encapsulated inside the liposome/DNA complexes and, therefore, better protected from enzymatic digestion. This was supported by Southern blot analysis: much more intact plasmid DNA was detected in tissues when MLV were used.

C. Charge Ratio

A recent study in this laboratory demonstrated that serum sensitivity of in vitro transfection can be overcome by increasing the charge ratio (+/–) between cationic lipid and DNA (90). Later studies showed that in vivo gene expression of either LPDI or cationic liposome/DNA complexes was also charge ratio dependent (63,64). As shown in Figure 6, increasing the amount of DOTAP resulted in a steady increase in gene expression in all tissues examined. It can be envisioned that some of the liposomes will stay as free liposomes at high +/– charge

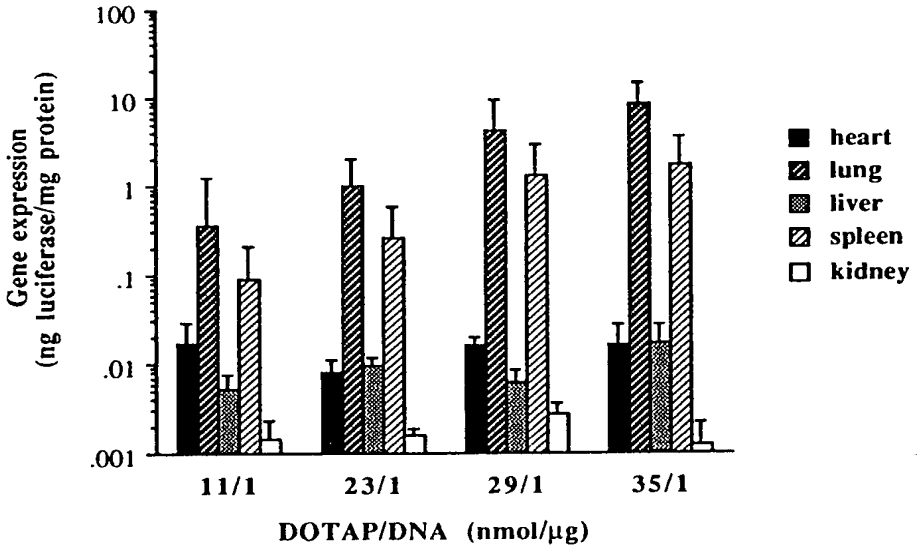


Figure 6 In vivo gene expression as a function of DOTAP concentration. pCMVL DNA was mixed with protamine (0.8 μg protamine/μg DNA) followed by the addition of various amounts of DOTAP liposomes. Fifty μg of DNA was injected into each mouse and gene expression was assayed 24 h later. The results was expressed as ng of luciferase per mg of protein (n = 3). (From Ref. 63 with permission.)

ratio. Excess cationic liposomes might help to protect the integrity of LPD by preventing the release of DNA from LPD by anionic molecules in the serum. Cationic liposomes also prolonged the residence of LPD in tissues, particularly in the lung. Taken together, these results provided a more efficient interaction of intact DNA with target cells, resulting in higher level of gene expression.

D. Lipid Compositions

The in vivo performance of cationic liposomes vary greatly with different cationic lipids and can not be well predicted from in vitro studies. For example, a lipid efficient in transfecting cells in vitro can show little activity in transfecting cells in vivo, and vice versa (53). The structural requirement of a cationic lipid for efficient intravenous gene delivery is not clear at present. The results of our in vivo studies with several different cationic lipids suggest that double-chain hydrocarbon-anchored lipids are more favorable for intravenous administration. Besides cationic lipids, selection of an appropriate helper lipid is also important for maximal in vivo gene expression. Several studies demonstrated that, in contrast to in vitro transfection, inclusion of DOPE resulted in a significant reduction in gene expression in vivo (62,65). Rather, cholesterol was shown to enhance the efficiency of in vivo gene transfer (62,65). Enhancement in gene expression by cholesterol might to due to its ability to stabilize the bilayer. A colloidally stable structure might be favorable for intravenous gene delivery. This might also partially explain why some double-chain hydrocarbon-anchored lipids are efficient in transfecting cells in vivo. Of course, other properties of a cationic lipid might also be important in determining its in vivo gene delivery efficiency.

It should be stated that while serum sensitivity of cationic lipidic vectors can be overcome by several different ways, current formulations are far from being well characterized. For example, MLV liposomes, although efficient, were more toxic than SUV liposomes (Li and Huang, unpublished data). Therefore, a vector should be characterized in the context of all important parameters including efficiency, toxicity, and stability. Another problem with these formulations is the difficulty of tissue-specific gene delivery. Intravenous gene delivery via cationic liposomal vectors is largely dependent on a first passage effect; lung is always the organ giving the highest level of gene expression and endothelial cells are the major cells transfected (63). Despite this limitation, there are many potential applications for systemic use of cationic liposome/DNA complexes. In these applications, delivery of gene to target cells via cationic lipidic vectors is achieved mainly through their intrinsic properties of non-specific interaction with pulmonary endothelial cells.

Few reports have been described of intravenous administration of neutral or anionic lipidic vectors for in vivo gene transfer (32,103) despite the fact that they are more compatible with the biological fluids. As mentioned above, the

major limitation on these vectors is the rapid clearance by RES. Development of long-circulating lipidic vectors, together with the use of a targeting ligand might provide a solution to the problem. It should be noted, however, that DNA is a large molecule with a large hydrodynamic diameter as compared with chemotherapeutic drugs. Therefore, the technology for formulating stealth liposomal drugs can not be directly used in the development of long-circulating lipidic gene vectors. Development of long-circulating lipidic vectors might require a more complicated process of self-assembling. For example, DNA can be first condensed by cationic lipids, polyamines, or lipopolyamines, followed by the incorporation of other favorable components. This possibility is currently being examined in several laboratories.

X. CONCLUSION

The last 10 years have seen great progress in the field of vector development for gene therapy. Nonviral vectors, particularly lipidic vectors, have grasped an increasing amount of attention. Many different lipidic vectors have been developed and several lipidic formulations are being used in clinical trials for the gene therapy of cancer and genetic diseases. Further studies on the transfection mechanism and lipid chemistry might result in the emergence of more active lipids and lipidic formulations. In addition, information from the clinical applications of lipidic vectors might provide new insights into the directions of future research. The vector could eventually be developed to be tissue-specific and, following receptor-mediated endocytosis, be more likely to undergo conformational changes and thus become more efficient in cytoplasmic delivery of DNA. Finally, advances in the understanding of gene expression in eukaryotic cells and the development of plasmid DNA with higher expression activity will help to improve the overall efficiency of gene therapy.

ACKNOWLEDGMENT

The original work in this laboratory was supported by NIH grants CA 64654, CA 71731, DK 44935 and a contract from Targeted Genetics Corporation.

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Functional Immunoliposomes Harboring a Biosynthetically Lipid-Tagged Single-Chain Antibody†

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Received June 6, 1994®

ABSTRACT: An anti-2-phenyloxazolone single-chain antibody was expressed in *Escherichia coli* as a lipoprotein fusion in order to generate a biosynthetically lipid-tagged molecule [Laukkanen et al. (1993) *Protein Eng. 6*, 449–454]. For purification, a hexahistidinyl tag was introduced to the C-terminus of the protein. The resulting antibody, termed Ox lpp-scFv-H6, was membrane-bound, displayed hapten-binding activity, and contained the lipoprotein-specific lipid modification as indicated by metabolic [^{3}H]palmitic acid labeling. The Ox lpp-scFv-H6 was purified by immobilized metal affinity chromatography followed by hapten-based affinity chromatography to essential homogeneity with a yield of 0.4–1.6 mg/L of culture. In detergent dialysis, the purified antibody partitioned quantitatively into phospholipid liposomes. The immunoliposome preparation consisting of a homogeneous population of unilamellar 100–200 nm vesicles displayed specific hapten-binding activity as measured by using ELISA and surface plasmon resonance (SPR)-based real-time biospecific interaction analysis. In SPR experiments, the immunoliposomes exhibited virtually irreversible binding to immobilized hapten compared to soluble antibody fragments, consistent with the predicted multivalent binding. Biosynthetic lipid-tagging of antibodies may prove useful for immunoliposome-based diagnostic and therapeutic applications.

Liposomes bearing antibody molecules on their surface have been shown to specifically bind to their appropriate molecular targets both *in vitro* (Gregoriadis & Neerunjun, 1975; Huang et al., 1980; Harsch et al., 1981; Martin et al., 1981) and *in vivo* (Hughes et al., 1989; Ahmad et al., 1993). This has aroused considerable interest into the use of immunoliposomes as vehicles for targeted drug delivery and for gene therapy (Heath et al., 1983; Wang & Huang, 1987; Maruyama et al., 1990). Moreover, immunoliposomes have been used in the design of novel formats for immunoassays (Kung et al., 1985; Plant et al., Pashkov et al., 1992) and as signal amplifiers in more conventional immunoassays (Ishimori & Rokugawa, 1993). Apart from these biotechnological applications, liposomes endowed with specific binding functions have been used as simplified model systems to study ligand–membrane receptor interactions (Egger et al., 1990; Lee et al., 1993).

Immobilization of antibody molecules to the surface of liposomes and planar lipid bilayers has been obtained through *in vitro* chemical conjugation to reactive lipid derivatives (Huang et al., 1980; Martin et al., 1981; Pinnaduwage & Huang, 1992; Lee et al., 1993). Because of their relative hydrophobicity, the fatty acylated antibody molecules associate stably with lipid membranes. Recent progress in the bacterial expression of functional antibodies as Fab fragments and as single-chain molecules (Hoogenboom et al., 1992; Plückthun, 1992; Skerra, 1993) prompted us to use genetic engineering to convert antibodies into membrane-bound molecules for immunoliposome applications. We expected that this approach would yield a defined product which can be effectively produced in bacteria and, at the same time, would obviate the tedious and potentially hazardous chemical treatments. We exploited the well-characterized machinery for the biosynthesis of *Escherichia coli* lipoproteins (Ichihara et al., 1981; Ghrayeb & Inouye, 1984; Choi et al., 1986; Gennity & Inouye, 1991) to design and produce a lipoprotein–single-chain antibody fusion protein (Laukkanen et al., 1993). We showed that this fusion antibody was fully active, was membrane-associated, and behaved like a membrane protein in detergent phase separation and in liposomal reconstitution experiments. In the present study, we describe the purification and reconstitution into liposomes of a biosynthetically lipid-tagged and polyhistidinylated anti-2-phenyloxazolone single-chain antibody and characterization of the immunoliposome–hapten interactions.

MATERIALS AND METHODS

Materials. Escherichia coli strain RV308 (su$^-$,Δ*lac*X74,*gal*-ISII::OP308,*str*A) was used as host for expression. Plasmids pKKtac and pML5 have been described earlier (Takkinen et al., 1991).

Egg yolk phosphatidylcholine, phosphatidylethanolamine, cholesterol, and *n*-octyl β-D-glucopyranoside were from Sigma. Triton X-100 was from Boehringer Mannheim. [9,10(N)-^{3}H]Palmitic acid (1.91 TBq/mmol) was purhased from Amersham. CNBr-activated Sepharose 4B and Chelating Sepharose Fast Flow were Pharmacia products. Lysozyme and DNA-modifying enzymes were products of Boehringer Mannheim and New England Biolabs. Sensor chips (CM5) and other reagents for BIAcore analysis were purhased from Pharmacia Biosensor AB.

Cloning of the Lipid-Tagged Antibody with a Hexahistidinyl Tail. Standard recombinant DNA techniques were used (Sambrook et al., 1989). The expression plasmid for the lipid-tagged antibody, pML3.7, contains the coding sequences for the signal peptide and nine N-terminal amino acid residues

† This work was partly supported by the Technology Development Centre of Finland (TEKES).

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® Abstract published in *Advance ACS Abstracts*, September 1, 1994.

0006-2960/94/0433-11664$04.50/0

of lpp fused to the anti-2-phenyloxazolone single-chain Fv[1] fragment (Laukkanen et al., 1993). A hexahistidinyl tail was introduced into this construct by using polymerase chain reaction (Saiki et al., 1988). Primers were 5'-GCGCCGACATCATAACGGTTC-3'(5'-primer, sense), which is complementary to the sequence in the promoter region of pKKtac, and 5'-AAGATAAGCTTCTAATGATGGTGATGATGATGTTTCAGCTCCAGCTTGGTCCCAGCACC-3' (3'-primer, antisense, nucleotides coding for histidine residues are underlined). The oligonucleotides were synthesized in Applied Biosystems 391 DNA synthesizer. The correctness of the 3'-modification was confirmed by DNA sequencing (Sanger et al., 1977), and the expression construct was designated as pML3.7H.

Purification of Lipid-Tagged Antibody. Bacterial expression of Ox lpp-scFv-H6 was carried out as described earlier (Laukkanen et al., 1993). Briefly, *E. coli* RV308 cells harboring plasmid pML3.7H were grown in TB medium (Sambrook et al., 1989) with 100 μg/mL ampicillin. An overnight culture was diluted 1:50 and grown to an OD_{600} of 1.5, and then isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. The induction was continued overnight at 30 °C.

Cells harvested from 1-L overnight culture were suspended in 50 mL of lysis buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.5 M NaCl, 0.1 mM PMSF, and 0.1 mg/mL lysozyme). After a 15-min incubation at room temperature, the lysis of the cells was completed by a brief sonication using a probe-type sonicator. The cell envelopes were collected by ultracentrifucation (150000*g*, 1 h, 4 °C). The pellet was suspended in buffer A [10 mM HEPES, pH 7.4, 1 M NaCl, 10% (v/v) glycerol, and 0.1 mM PMSF] and recentrifuged as above. The pellet was resuspended in 15 mL of buffer A containing 1% (w/v) Triton X-100. The solubilization was carried out overnight at 4 °C with continuous rotation, and cleared by ultracentrifugation (150000*g*, 1 h, 4 °C).

For immobilized metal affinity chromatography (IMAC; Porath & Olin, 1983; Hochuli et al., 1988; Smith et al., 1988), the solubilized preparation was diluted 1:5 in buffer A. The final concentration of Triton X-100 was 0.2% (w/v) at this stage. The sample was incubated overnight at 4 °C in 50-mL total volume and constant mixing with 0.5–1 mL of Chelating Sepharose Fast Flow charged with Ni^{2+} as described previously (Porath & Olin, 1983). To decrease the binding of endogenous *E. coli* proteins, 1 mM imidazole was included in the buffer. The resin was loaded into a column and washed in a stepwise manner with 10 bed volumes each of buffer A containing 0.2% (w/v) Triton X-100 and 1, 50, 75, 100, and 250 mM imidazole.

For hapten-based affinity chromatography, BSA containing approximately 21 molecules of oxazolone, Ox_{21}BSA (Mäkelä et al., 1978), was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. The pooled 75 and 100 mM imidazole fractions containing most of the Ox lpp-scFv-H6 were diluted 1:5 in buffer A containing 0.2% (w/v) Triton X-100 and incubated with Ox_{21}BSA resin in suspension as above. Washing and elution steps in hapten-based affinity chromatography were performed in a column as described previously (Takkinen et al., 1991; Harlow & Lane, 1988) except that 0.2% (w/v) Triton X-100 or 1% (w/v) *n*-octyl β-D-glucoside (OG) was present in the buffers.

Preparation of Liposomes. Pure egg yolk phospholipid/cholesterol mixture (10 mg, PC/PE/Cho, 10:1:5 molar ratio) was dissolved in 5 mL of 1% (w/v) OG with or without 40 μg of the purified Ox lpp-scFv-H6. The detergent was removed by dialyzing against 10 mM HEPES (pH 7.4) either overnight in cellulose dialysis bags (cutoff 12–14 kDa) with two buffer changes or in a LIPOSOMAT dialyzer (Dianorm, Munich, Germany) with cellulose membranes (cutoff 10 kDa). After detergent removal, the liposomes were collected by ultracentrifugation (150000*g*, 1 h, 4 °C) and suspended in 5 mL of 10 mM HEPES (pH 7.4).

Surface Plasmon Resonance Analysis of Liposomes. Binding properties of liposomes to the hapten were also characterized by using the BIAcore instrument (Pharmacia Biosensor AB, Uppsala, Sweden) employing the surface plasmon resonance (SPR) phenomenon (Löfås et al., 1991; Sjölander & Urbaniczky, 1991). The result of the measurement is converted into a sensorgram which shows the time development of the arbitrary resonance unit (RU) value which relates to the change in the incident angle of total internal reflection giving rise to SPR and is proportional to the mass interacting with the evanescent wave field in the dextran layer. A sensor chip (CM5) with a carboxymethylated dextran layer was used to immobilize Ox_{16}BSA and BSA. The immobilization was carried out according to the manufacturer's instructions using the Amine Coupling kit (Pharmacia Biosensor AB). Briefly, 35 μL of 75 μg/mL Ox_{16}BSA or 5 μg/mL BSA in 10 mM sodium acetate buffer (pH 4.0) was injected into carboxymethylated dextran activated with 35 μL of a 1:1 mixture of *N*-hydroxysuccinimide (0.1 M) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (0.4 M). Finally, the remaining activated carboxyl groups were blocked with injection of 35 μL of ethanolamine hydrochloride (1 M, pH 8.5). Noncovalently bound protein was removed by injecting 20 μL of 100 mM HCl. Immobilization levels of Ox_{16}BSA and BSA were approximately 2000 and 5000 resonance units (RU), respectively. The immobilization and the subsequent binding analyses were performed under a constant flow rate of 5 μL/min of 10 mM HEPES (pH 7.4), 3.4 mM EDTA, and 0.15 M NaCl. Liposome samples (30 μL of a 1:3 dilution unless otherwise stated) were injected onto the sensing surface followed by a buffer flow. Regeneration of sensor chips was performed with 50 μL of 0.5% (w/v) Triton X-100.

Electron Microscopy. The liposome samples were concentrated 10-fold by ultracentrifugation (150000*g*, 1 h, 4 °C) and resuspension in 10 mM HEPES, pH 7.4. A drop of liposomes was dried on a carbon-coated copper grid (150–200 mesh) and stained with 1% potassium phosphotungstate, pH 7.4. Negative-stained liposomes were observed with a Jeol JEM-100CX transmission electron microscope at 60 V.

Other Methods. Protein samples were analyzed by SDS–PAGE according to Laemmli (1970). Metabolic [^{3}H]palmitic acid labeling and immunoblotting using a polyclonal antiserum raised against the parental Ox scFv were otherwise carried out as described earlier (Laukkanen et al., 1993; Towbin et al., 1979). Hapten-binding activity measurements using ELISA were performed as described previously (Laukkanen et al., 1993). Protein content was determined by using a modified Amido Black method (Kaplan & Petersen, 1989).

RESULTS

Bacterial Expression of Lipid-Tagged Antibody with a Hexahistidinyl Tail. Our initial attempts in using hapten-

[1] Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Fab, antigen-binding fragment (Fd and L chains); Fv, variable region fragment; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; scFv, single-chain Fv; SDS, sodium dodecyl sulfate; TB, terrific broth.

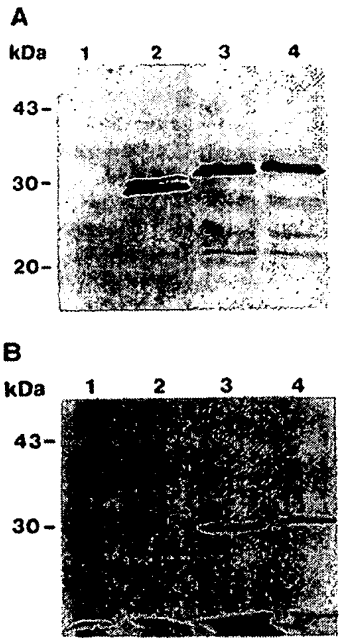

FIGURE 1: Bacterial expression of Ox lpp-scFv-H6. Whole-cell samples were analyzed by SDS–PAGE (15%) followed by immunoblotting using anti-Ox scFv antiserum (A) or by fluorography (B). *E. coli* RV308 cells harboring the plasmid pKKtac (vector control, *lanes 1*), pML5 (expression of the soluble Ox scFv, *lanes 2*), pML3.7 (expression of Ox lpp-scFv, *lanes 3*), pML3.7H (expression of Ox lpp-scFv-H6, *lanes 4*) were induced with IPTG for protein expression in the absence (A) or presence (B) of [^{3}H]palmitate.

based affinity chromatography to purify the lipid-tagged antibody, Ox lpp-scFv, from the crude detergent extract of bacterial membranes were unsuccessful. Therefore, we introduced a stretch of six histidinyl residues into the C-terminus of the antibody to serve as an affinity tag in immobilized metal affinity chromatography (IMAC). The resulting construct, pML3.7H, was expressed in *E. coli* RV308 as a 30-kDa species recognized by the antiserum raised against the purified soluble Ox scFv (Figure 1A). The slightly larger size of Ox lpp-scFv-H6 as compared to Ox lpp-scFv (28 kDa) which lacks the polyhistidinyl tag is consistent with the expected contribution of six weakly basic histidinyl residues to the electrophoretic mobility. The Ox lpp-scFv-H6 antibody was cell- and membrane-bound since very little if any immunoreactive Ox lpp-scFv-H6 was detected in the culture supernatant (data not shown). This is in good agreement with our earlier results showing the tight membrane association of Ox lpp-scFv mediated by the N-terminal lipid tag (Laukkanen et al., 1993). In metabolic labeling experiments, [^{3}H]-palmitate was incorporated into a 30-kDa species present in Ox lpp-scFv-H6-expressing cells but not in control cells expressing the soluble antibody (Figure 1B), indicating the biosynthetic incorporation of fatty acid into the lpp-fusion antibody.

Purification of Lipid-Tagged Antibody. The fusion protein was solubilized from the bacterial cell envelopes in 1% (w/v) Triton X-100. More than 95% of the solubilized Ox lpp-scFv-H6 was bound to Ni^{2+}-charged Chelating Sepharose as determined by ELISA (data not shown). The elution was performed in a stepwise manner with increasing imidazole concentrations. Most of the antibody eluted with buffers containing 75 and 100 mM imidazole. Silver-stained SDS–PAGE of the eluted material showed a major 30-kDa band and several minor bands of smaller size (Figure 2, *lane 4*).

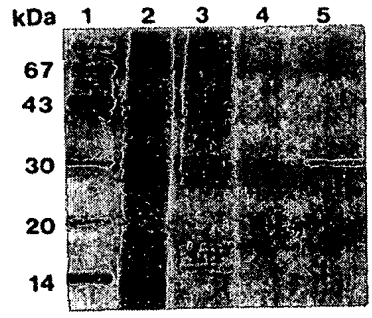

FIGURE 2: Purification of Ox lpp-scFv-H6. The silver-stained SDS–polyacrylamide gel (15%) shows the protein pattern at different stages of purification. Lanes: *1*, molecular mass markers; *2*, whole cell lysate; *3*, Triton X-100 extract; *4*, pooled 75–100 mM imidazole eluates from the Ni^{2+} chelating Sepharose column; *5*, low-pH eluate of the Ox_{21}BSA–Sepharose affinity column.

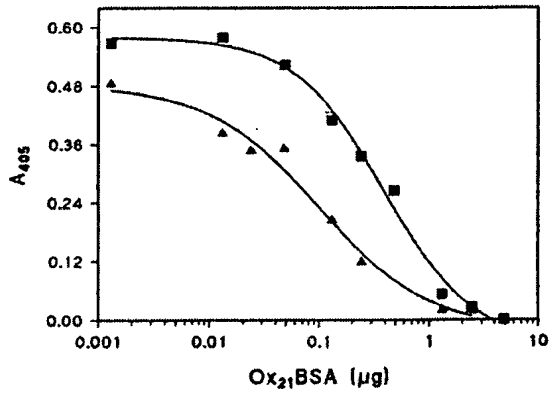

FIGURE 3: Hapten-binding activity of purified Ox lpp-scFv-H6. Purified Ox scFv (0.6 μM, ▲) and Ox lpp-scFv-H6 (1.1 μM, ●) and different amounts of soluble hapten (Ox_{21}BSA) were analyzed by ELISA as described earlier.

The lipid-tagged antibody was further purified by using hapten-based affinity chromatography in which Triton X-100 was exchanged to octyl glucoside for later reconstitution experiments. From 80% to 95% of the hapten-binding activity in the pooled 75–100 mM imidazole fractions was retained by an Ox_{21}BSA–Sepharose affinity column and subsequently eluted in low-pH buffer (100 mM glycine hydrochloride, pH 2) in the presence of OG. The purified antibody migrated as a single 30-kDa species in SDS–PAGE (Figure 2, *lane 5*). The recovery of the purified protein varied from 0.4 to 1.6 mg from a 1-L culture. The hapten-binding activity of the purified antibody was measured in ELISA, where the binding of Ox lpp-scFv-H6 and the parental Ox scFv to immobilized Ox_{21}-BSA was inhibited by soluble hapten at the same concentration range, indicating the absence of any major differences between these two antibody forms (Figure 3). The purified lipid-tagged antibody retained 66–75% of the hapten-binding activity after a 1-week incubation at room temperature or at 4 °C (Table 1).

Preparation of Immunoliposomes. The purified Ox lpp-scFv-H6 was reconstituted into phospholipid/cholesterol (PC/PE/Cho, 10:1:5 molar ratio) liposomes by removal of detergent in dialysis. The reconstitution mixture was then subjected to ultracentrifugation, and the resulting liposome pellet and supernatant were analyzed by immunoblotting (Figure 4A). No immunoreactivity (or hapten-binding activity) was detected in the supernatant (*lane 1*), whereas the 30-kDa Ox lpp-scFv-H6 band was quantitatively recovered in the liposome pellet

Table 1: Stability of Purified Lipid-Tagged Antibody and Immunoliposomes[a]

sample	A_{405} control	4 °C	RT	37 °C
purified Ox lpp-scFv-H6	0.550	0.416	0.366	0.010
immunoliposomes	0.341	0.374	0.166	0.074

[a] Values are means of duplicate measurements. The hapten-binding activities were measured by using ELISA. Comparison of A_{405} values between a freshly made sample (control) and samples after a 1-week incubation in different temperatures is presented. No significant binding to BSA was observed.

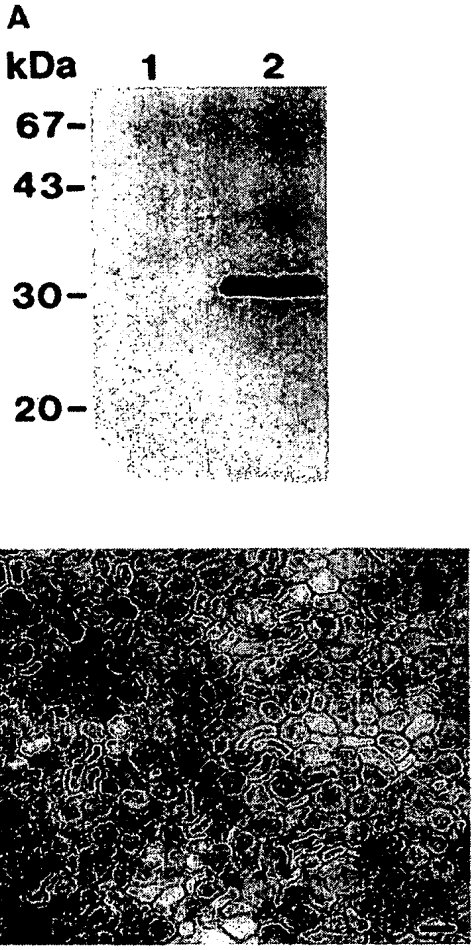

FIGURE 4: Reconstitution of Ox lpp-scFv-H6 into liposomes. (A) Immunoblot of the supernatant (lane 1) and liposome pellet (lane 2) of the ultracentrifuged reconstitution mixture. (B) Electron micrograph of the negative-stained liposome pellet (bar, 200 nm).

(*lane 2*), indicating efficient incorporation of the antibody into liposomes. In a negative-stained electron micrograph, the liposome preparation appeared as a relatively homogeneous population of vesicular structures with 100–200 nm diameter (Figure 4B). No signs of multilamellar structures were evident in these or in freeze–fracture (data not shown) EM pictures. The appearance of the immunoliposomes analyzed by EM is in keeping with the known unilamellarity and size range of liposomes prepared from octyl glucoside solutions (New, 1990). We calculated the average number of antibody molecules per liposome to be about 2000 by using the size and contents of protein and lipid in the immunoliposomes and by assuming full recovery. Half of the antibody molecules would be on the outer surface as the detergent dialysis method is expected to lead to a 50/50 distribution in terms of inward/outward orientation of the reconstituted protein. In recent experiments, we have reconstituted the lipid-tagged antibody to premade liposomes by diluting the antibody preparation in octyl glucoside directly into a large excess (10–20× volume) of liposomal suspension followed by overnight incubation and ultracentrifugation. Also, this approach leads to efficient incorporation of the antibody into the liposomes (data not shown).

Functional Characterization of Immunoliposomes. The hapten-binding activity of the immunoliposomes was analyzed by using BIAcore which monitors in real time small changes in the refractive index of the sensing layer caused by association/dissociation of macromolecules to a derivatized dextran matrix encased in a sensor chip (Löfås et al., 1991; Sjölander & Urbaniczky, 1991). Figure 5A shows a sensorgram of the binding of Ox lpp-scFv-H6-containing liposomes to Ox_{16}BSA during the 6-min injection of the liposome preparation (steps *1–3*). After the association phase (step *2*), the sample is replaced by buffer flow (steps *3–5*), and finally the sensor surface is regenerated with Triton X-100 (step *5*). In contrast to the binding of the immunoliposomes, the liposomes lacking the antibody did not bind to Ox_{16}BSA (Figure 5B). However, the immunoliposomes displayed low but significant binding to BSA (Figure 5B) which was not affected by the presence of soluble hapten (data not shown). The absence of any apparent dissociation of the immunoliposomes from Ox_{16}BSA contrasts the relatively rapid dissociation of soluble Ox scFv and is consistent with the multivalent nature of the hapten–antibody interaction in the immunoliposomes (Figure 5C). The binding of the immunoliposomes is mediated by the specific hapten–antibody interaction as it is displaced in a concentration-dependent manner by soluble hapten (Figure 6). By using BIAcore, the binding studies of purified Ox lpp-scFv-H6 to immobilized hapten in the presence of detergent (Triton X-100 and OG) have been unsuccessful, most likely due to optical interference of detergent micelles with the measurement.

The stability of the immunoliposomes was studied by incubating the immunoliposomes in HEPES buffer at different temperatures followed by ultracentrifugation and hapten-binding activity measurements in ELISA. No loss of hapten-binding activity was observed after an 8-day incubation at 4 °C whereas in samples incubated at room temperature and at 37 °C activity losses of 50% and 80%, respectively, were observed (Table 1). The partial loss of activity at room temperature and at 37 °C was not associated with the release of the lipid-tagged antibody from the liposomes or with major proteolytic degradation as evidenced by immunoblots of the supernatant and liposome pellets (data not shown).

DISCUSSION

The recent advances in bacterial expression of antibodies and in phage display technology now allow rapid isolation of desired antibodies and their large-scale production in *E. coli* as functional Fab and Fv fragments and as single-chain Fv fragments (Barbas, 1993; Skerra, 1993). The successful use of phage antibodies and bacterially expressed antibody fragments as immunological reagents has been demonstrated and is expected to largely replace the hybridoma-based monoclonal antibody technology (Nissim et al., 1994). By further engineering of antibody fragments, it is possible to introduce tags for easy detection and purification or to drive dimerization for higher avidity (Pack & Plückthun, 1992;

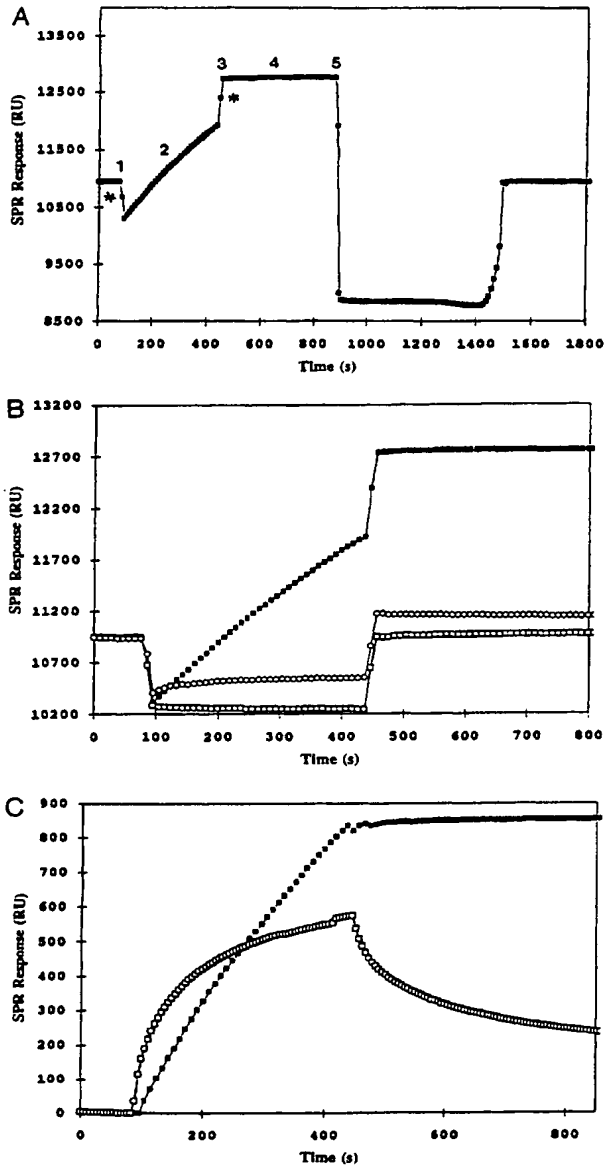

FIGURE 5: SPR analysis of immunoliposome binding. (A) A BIAcore sensorgram showing the injection of immunoliposomes (1) and the association phase (2), and after injection, the sample is replaced by buffer flow (3) and the apparent dissociation phase (4) followed by regeneration (5) of the sensor surface. The asterisk shows the bulk effect caused by the liposome suspension. (B) Binding of immunoliposomes (■) and liposomes without protein (□) to Ox_{16}BSA and binding of immunoliposomes (○) to BSA only. (C) A sensorgram of binding of 1:6 diluted immunoliposomes (■) and 600 nM Ox scFv (□) to Ox_{16}BSA. The data in panel C are corrected for the bulk effect.

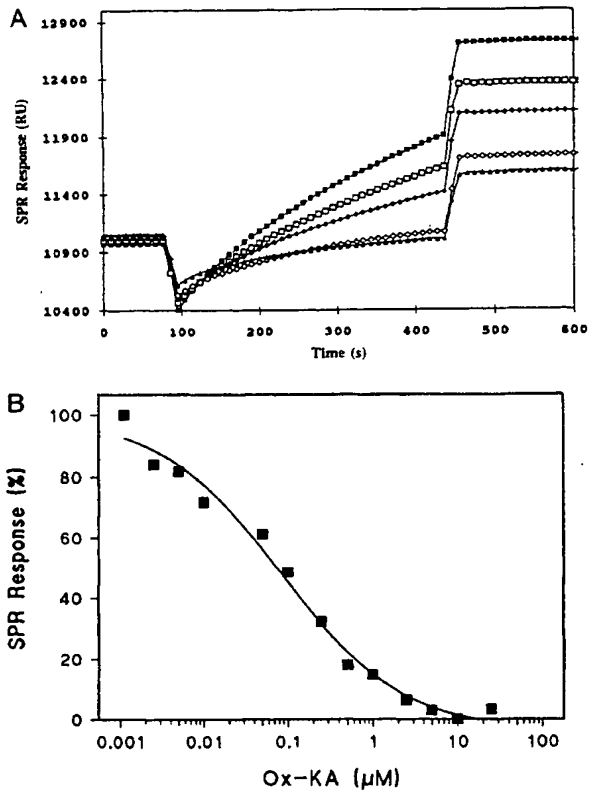

FIGURE 6: Specificity of immunoliposome binding in the SPR measurement. Immunoliposomes were injected in the presence of increasing concentrations of a soluble hapten, caproic acid conjugate of 2-phenyloxazolone (Ox-KA). (A) Overlay plot of sensorgrams obtained with immunoliposome samples containing 0.001 (■), 0.01 (□), 0.1 (♦), 1 (◇), and 10 μM (▲) Ox-KA. (B) Decrease of the specific SPR response as a function of Ox-KA concentration.

Skerra et al., 1991; Schmidt & Skerra, 1993). Furthermore, whole proteins or functional domains have been fused to bacterially expressed antibodies to generate hybrids with alkaline phosphatase (Wels et al., 1992; Ducancel et al., 1993), protein kinase (Ueda et al., 1992), protein A (Tai et al., 1990), or metallothionein (Das et al., 1992). These antibody fusion proteins may find use in immunoassays, immunotherapy, and medical imaging.

An obvious possibility to further increase the practical utility of bacterially expressed antibodies is to introduce tags to provide oriented immobilization to different matrices. We described earlier the bacterial expression of a single-chain antibody fused to the signal sequence and nine N-terminal amino acids of *E. coli* major lipoprotein (lpp) (Laukkanen et al., 1993). This design leads to the biosynthetic incorporation of a lipid moiety into the antibody and anchoring of the antibody stably to the bacterial membrane. The lipid-tagged antibody can be solubilized in detergents and transferred to proteoliposomes with retention of the hapten-binding activity.

The purification of the bacterially expressed and membrane-bound lpp–antibody fusion by hapten affinity chromatography proved difficult. This was probably due to the interference of the complex bacterial detergent extract with the purification scheme developed earlier for a soluble antibody fragment (Takkinen et al., 1991). Encouraged by earlier work (Skerra et al., 1991), we subsequently introduced a stretch of six histidines into the C-terminus of the lipid-tagged single-chain antibody to serve as an affinity tag in IMAC. The tag worked remarkably well, facilitating substantial purification of the Ox lpp-scFv-H6 from the detergent extract of bacterial membranes. The minor contaminants in the preparation were then removed by hapten-based affinity chromatography. Without any optimization, the recovery of purified antibody from 1 L of shake flask culture was about 1 mg. It is likely that substantially higher yields can be achieved by using high cell density fermentation (Pack et al., 1993).

The rationale of our work is to develop a tag for stable and oriented immobilization of purified antibodies to lipid mem-

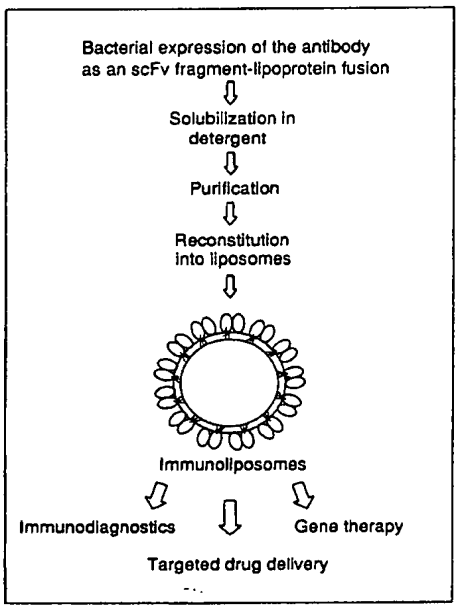

FIGURE 7: Biosynthetic lipid-tagging of antibodies. A schematic presentation showing the principal and major technical steps involved. See the text for more details.

branes. Here, the high potency of the biosynthetically generated lipid tag for immobilization was demonstrated by the very efficient incorporation of the purified lipid-tagged antibody into phospholipid liposomes by using detergent dialysis as judged by immunoblotting. Importantly, adsorption of the lipid-tagged antibody directly to premade liposomes was also possible. This method is more compatible with applications which involve encapsulation of water-soluble material with the liposomes, for which sonication and high-pressure extrusion methods are widely used (Olson et al., 1979; Hope et al., 1985). The immunoliposomes displayed specific hapten-binding activity in an SPR-based assay. The binding was inhibited by soluble hapten (caproic acid conjugate) in a concentration-dependent manner. This shows that the immunoliposomes harboring a biosynthetically lipid-tagged single-chain antibody are able to recognize and bind to the appropriate molecular target. Regarding the use of immunoliposomes as immunological reagents or as vehicles for drug delivery, the strength of the antibody–antigen interaction mediating the binding of liposomes to the antigen is important. Multivalent binding, obtained through the presence of many antibody molecules in the liposome, is expected to lead to higher binding avidity. The very slow dissociation that we observed in BIAcore experiments is in good agreement with the expected multivalent binding of immunoliposomes.

Presently, there is considerable interest in the use of liposomes as efficient and well-tolerated drug carriers for *in vivo* applications (Gregoriadis & Florence, 1993). Specific binding of immunoliposomes charged with drugs, DNA, or imaging agents may be exploited in the design of novel therapeutic and diagnostic approaches. Furthermore, use of immunoliposomes as specific reagents in various kinds of immunoassays has been described. The practical utility of bacterially produced lipid-tagged antibodies for any of the above-mentioned applications still remains to be demonstrated. However, we envision that the biosynthetic lipid-tagging approach as described herein [Figure 7; see also Keinänen and Laukkanen (1994)] may become an alternative to the presently used chemical conjugation methods for functional immobilization of antibodies to liposomes for targeted drug delivery and immunodiagnostic and other applications. In conclusion, the present study demonstrates the bacterial expression, purification, and functional reconstitution into liposomes of a lipoprotein–single-chain antibody fusion molecule, illustrating a novel method for stable and oriented immobilization of antibodies to lipid bilayers.

ACKNOWLEDGMENT

We thank Drs. Hans Söderlund and Kristiina Takkinen for their valuable comments and discussions. We also gratefully acknowledge the expert technical assistance of Anja Pallas and Oili Lappalainen. We thank the Department of Electron Microscopy, University of Helsinki, for providing EM facilities.

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